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**IDENTIFYING THE MEROZOITE TARGETS OF  
PROTECTIVE IMMUNITY TO *PLASMODIUM FALCIPARUM*  
MALARIA**

A thesis submitted to the Open University for the degree of  
DOCTOR OF PHILOSOPHY  
BIOLOGICAL SCIENCES

**Affiliated Research Centre**

KEMRI-Wellcome Trust Research programme, Kilifi, Kenya

**Collaborating Establishment**

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## ABSTRACT

The observation that individuals living in malaria endemic regions who are repeatedly infected with *P. falciparum* can acquire immunity, first to severe, then to uncomplicated clinical episodes of malaria, and finally to high parasite densities, provides hope that a vaccine is achievable. Immunoglobulins have been identified as a key component of naturally acquired immunity and identifying the targets and mechanisms by which this protection is achieved is a clear research priority. To date, only a small proportion of the parasite proteome has been evaluated in the context of naturally acquired immunity. This thesis was aimed at contributing to this knowledge gap by identifying novel potential antigen targets of protective antibodies and validating these in samples from Tanzanian adults.

First, to identify merozoite antigens that were immunogenic, I resolved proteins extracted from *P. falciparum* merozoites by two-dimensional gel electrophoresis and tested these for reactivity with immunoglobulins from malaria immune adults. Immunoreactive proteins were then identified by mass-spectrometry. In complementary studies, purified *P. falciparum* merozoites were treated with proteolytic enzymes to release proteins localised on the surface of merozoites, which were subsequently identified by mass-spectrometry. Using stringent criteria, where I combined the data obtained from 2D-immunoblots and surface-trypsinization experiments with bioinformatics prediction (for the presence of a signal peptide and/or transmembrane domain), I narrowed down to 222 potential merozoite vaccine targets. These included known surface and/or immunogenic proteins such as the 6-cysteine proteins (*Pf*12, *Pf*38, *Pf*41), MSP-1, 3, 7, 9, 10, GLURP, AMA1, GAMA, MTRAP, LSA3 and RhopH3 as well as many unstudied proteins. From the set of unstudied proteins, I prioritised 27 antigens for immunoprofiling and identified 19 antigens that are targets of naturally acquired antibodies and potential novel vaccine candidates.

Next, using a cohort of adults living in a village in Tanzania that experiences hyperendemic malaria transmission throughout the year, I examined antibody

responses to the novel potential vaccine candidates to test whether they were correlated with protective immunity. I began by identifying a panel of antigens that were immunogenic and elicited a stable antibody response in adults. Subsequently, I identified six antigens that were individually associated with protection from clinical episodes of malaria. Individuals who became ill during the follow-up period had significantly lower levels of these antibodies compared to those who did not. These antigens were the pantothenate transporter (*PfPAT*), a putative amino acid transporter (PF3D7\_0629500), PF3D7\_0830500, PF3D7\_1025300, PF3D7\_1345100 and PF3D7\_1401600. In addition, the breadth of antibody responses to the tested antigens was associated with protection from clinical malaria. Finally, four of these six antigens strongly correlated with protective effector functions. Antibody responses to *PfPAT* were strongly correlated to both the ability to fix soluble factor C1q (C1q-fixation) to merozoites as well as with their interaction with neutrophils to release reactive species (ADRB). In addition, antibody responses to the putative amino acid transporter, PF3D7\_1345100 and PF3D7\_1401600 were strongly associated with C1q-fixation ability. Recruitment of the soluble factor C1q onto the surface of merozoite results in lysis via the classical complement pathway. The release of reactive oxygen species by neutrophils is thought to be toxic to the intra-erythrocytic stages of the parasites and is associated with parasite clearance.

This thesis shows that 19 antigens, some of which have been studied for the first time in this work, are targets of naturally acquired antibody responses. Six of these antigens appeared to be associated with protective immunity to malaria in adults and correlated strongly with immune effector mechanisms that are thought to be important for parasite clearance. These findings provide a set of antigens that warrant further evaluation for inclusion into the vaccine pre-clinical development pipeline.



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## **DEDICATION**

**I dedicate this thesis to the memory of Cúcú and Gúka, Felistas Gathoni and  
Patrick Kamere; may they rest in peace**

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## **LIST OF ABBREVIATIONS**

ACT: Artemisinin-based Combination Therapy  
ADRB: Antibody-dependent respiratory burst  
ADCI: Antibody-dependent cellular inhibition  
AIDS: Acquired Immune Deficiency Syndrome  
CR1: Complement receptor 1  
CIDR: Cysteine rich inter-domain regions  
CSA: Chondroitin Sulfate A  
CSP: Circumsporozoite protein  
CelTOS: Cell-traversal protein for ookinetes and sporozoites  
DARC: Duffy antigen receptor for chemokines  
DBL: Duffy binding-like  
DBP: Duffy binding protein  
EIR: Entomological inoculation rate  
EBL: Erythrocyte binding-like  
EXP: Exported protein  
EPCR: Endothelial protein C receptor  
HbC: Hemoglobin C  
HbS: Hemoglobin S  
HbE: Hemoglobin E  
HIV: Human Immunodeficiency virus  
HRP: Histidine Rich Protein  
ICAM-1: Intercellular adhesion molecule 1  
ITN: insecticide treated nets  
IRS: Indoor residual spraying  
IPT: Intermittent preventive treatment  
G6PD: Glucose-6-phosphate dehydrogenase  
GTS: Global Technical Strategy  
GPI: Glycosylphosphatidylinositol  
LSA: Liver stage antigen



MSP: Merozoite surface protein  
NAI: Naturally Acquired Immunity  
OPA: Opsonic-phagocytosis assay  
PAM: Pregnancy associated malaria  
PfEMP1: *Plasmodium falciparum* erythrocyte membrane protein 1  
PK: Pyruvate Kinase  
PVM: Parasitophorous Vacuole Membrane  
RDT: Rapid Diagnostic Test  
RIFIN: Repetitive interspersed family  
SAO: Southeast Asian Ovalocytosis  
SEA: Schizont Egress Antigen 1  
SMFA: Standard membrane feeding assays  
STARP: Sporozoite Threonine and Asparagine-Rich Protein  
SMC: Seasonal malaria chemoprevention  
SP: Sulphadoxine-pyrimethamine  
SURFIN: Surface-associated interspersed gene family  
STEVOR: Sub-telomeric variable open reading frame  
sSA: sub-Saharan Africa  
TRAP: Thrombospondin related anonymous protein  
UI: Uncertainty Intervals  
VSA: Variant surface antigens  
WHO: World Health Organisation

# CHAPTER 1

## Introduction

### 1.0. Malaria: Burden of disease

Malaria is a life-threatening disease characterised by an acute febrile illness, which was estimated by the World Health Organisation (WHO) to result in 212 million cases (uncertainty interval (UI): 148-304 million) and 429,000 deaths (UI: 235,000-639,000) in 2015 [1]. Within the last one and a half decades however, there have been major gains in the control of the disease, which has resulted in an estimated drop by 21% and 29% in the malaria incidence rate and mortality, respectively, between 2000 and 2015 [1]. Despite these gains, 91 countries in sub-Saharan Africa, South-East Asia, Latin America and the Middle East continue to experience malaria transmission and individuals in these regions remain at risk [1]. The largest share of the global burden of malaria is experienced in Sub-Saharan Africa accounting for 90% and 92% of the clinical cases and deaths respectively. Southeast Asia and the Middle East accounted for 7% and 2% of the clinical cases reported in 2015, respectively [1].

Five *Plasmodium* parasite species namely: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* cause malaria in humans. The majority of deaths (99%) due to malaria are caused by *P. falciparum*, although *P. vivax* is the dominant infective species outside Africa. Infection with *P. knowlesi* also results in severe disease and death, however transmission is limited to South-East Asia [2]. The symptoms following infection with *P. malariae* and *P. ovale* are often mild and rarely result in death. In individuals living in malaria endemic regions, the burden of disease occurs primarily in children aged 5 years and below. The WHO estimated that 70% or 303,000 (UI: 165,000-450,000) of the total deaths due to malaria in 2015 occurred in this age group [1]. In addition, some populations in endemic regions are at particular risk, including pregnant women, HIV-infected individuals and non-immune individuals.

Several malaria control strategies are available that have contributed to the decline in the burden of disease and are discussed in detail in section 1.3 of this chapter. These strategies are primarily aimed at: i) vector control, ii) preventing disease in “at-risk” populations through prophylaxis, iii) rapid and accurate diagnosis and treatment of malaria and iv) continued malaria surveillance. So far, a highly effective vaccine against malaria has been elusive but pilot studies on the feasibility of the roll-out of the RTS’S vaccine are set to begin in 2018 offering some hope [1, 3]. These control measures continue to be employed with the aim of achieving the three main goals that were set by the Global Technical Strategy for malaria between 2016-2030 (GTS): i) To reduce the malaria incidence and mortality rates by 90% by the year 2030, ii) eliminate malaria from 35 countries by 2030 iii) prevent re-establishment of malaria in countries that are malaria free [1]. To achieve these goals, there is no doubt that an effective vaccine is urgently needed.

### **1.1. *Plasmodium* spp life cycle**

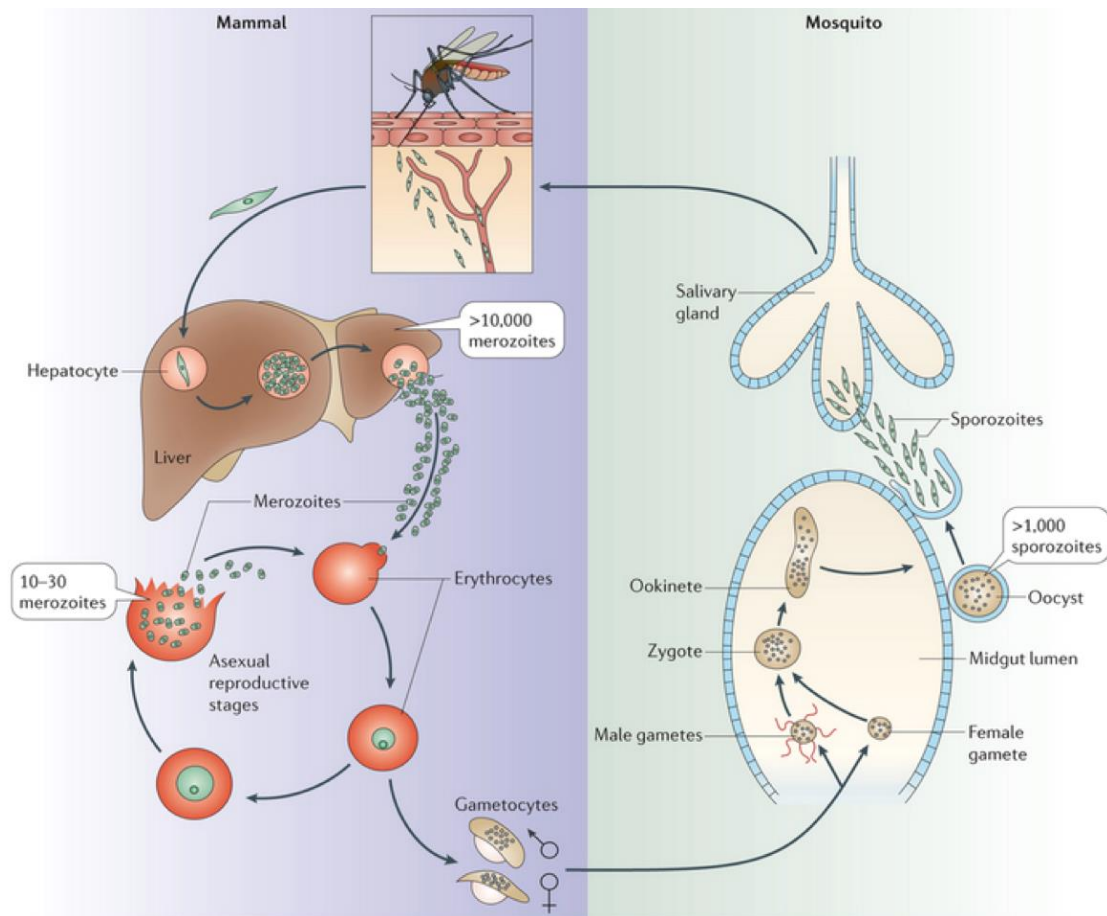
Malaria infection in humans is caused by intracellular obligate parasites of the *Plasmodium* spp. that are transmitted via the bite of an infected female mosquito of the *Anopheles* genus. All the five human infective *Plasmodium* species have a similar life cycle that requires two hosts: the mosquito vector and the vertebrate host as shown in Figure 1.1 [4]. However, there are subtle differences in the length of the life cycle and in the parasite morphology at various developmental stages between the five species [5].

As the female *Anopheles* mosquito takes a blood meal it injects around 10-100 motile sporozoites into the dermis. These sporozoites first migrate to the capillaries, and then travel via the bloodstream to the liver. Sporozoites cross the liver sinusoidal cell barrier by the transversal of either endothelial cells or Küpffer cells (phagocytic cells resident in the liver) and migrate through several cells finally invading a hepatocyte where their subsequent development occurs. After hepatocyte invasion, the sporozoite is enclosed within a parasitophorous vacuole membrane

(PVM), which forms a physical barrier from the hepatocyte's cytoplasm. Enclosed within the PVM, sporozoites undergo asexual development and replication, amplifying their numbers exponentially and developing into pre-erythrocytic schizonts that contain merozoites. One sporozoite developing within a hepatocyte can develop into 40,000 merozoites [6]. These merozoites are packaged within host membrane-surrounded vesicles called merosomes that bud off hepatocytes and are released into the blood circulation. This clinically silent phase is referred to as the pre-erythrocytic stage of the life cycle and lasts about 6-9 days for *P. falciparum* infection [4]. Pre-patent periods for *P. vivax*, *P. ovale*, *P. malariae* are 8-12, 10-14 and 15-18 days respectively. It has been estimated that 40,000, 10,000, 15,000 and 2,000 merozoites are produced per sporozoite during the pre-erythrocytic stage in *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* infections respectively [7]. In *P. vivax* and *P. ovale* infections, some of the sporozoites do not immediately undergo asexual replication but enter a dormant phase and are called hypnozoites that can cause relapses weeks or months after an initial infection[8].

After being released from merosomes into the blood circulation, merozoites attach to and invade red blood cells, initiating the erythrocytic stage of the life cycle. Merozoites develop within the erythrocytes through specific stages, termed the ring, trophozoite and schizont stages that are morphologically distinct by light microscopy. The young trophozoite is referred to as the ring form due to its morphology on Giemsa staining. As the trophozoite enlarges and changes in morphology, the parasite ingests the host cytoplasm and breaks down haemoglobin as a source of amino acids and energy. A by-product of this process, haemozoin, is visible as golden-brown to black crystalline granules in the mature stages. Nuclear division begins in the schizont stage followed by a budding process in mature schizonts where individual merozoites become visible resulting in erythrocytes containing segmented schizonts. These schizonts rupture releasing newly formed merozoites into circulation that can invade other erythrocytes resulting in a cyclic blood-stage infection. This stage takes approximately 48 hours for *P. falciparum*, *P. vivax* and *P. ovale* infections, 24 hours for *P. knowlesi* and 72 hours for *P. malariae*

infections [5]. Triggered by poorly understood cues, a small proportion of newly invaded merozoites can differentiate into sexual gametocytes that after a series of developmental stages can be taken up by the *Anopheles* mosquito. The male and female gametocytes exit erythrocytes and undergo nuclear replication forming gametes within the mosquito mid-gut. The male gamete (microgamete) is morphologically distinct from the female gamete (macrogamete) as they contain flagella that render them motile. Within the midgut, the gametes fuse to form a zygote (diploid) that differentiates into a motile form referred to as an ookinete. The ookinete transverses the mosquito midgut wall forming oocysts within which haploid sporozoites develop. The oocysts rupture releasing newly formed sporozoites that travel through the haemocoel to invade the salivary glands of the mosquito from where they can then be transmitted to the human host [4].



**Figure 1.1: The life cycle of the *Plasmodium* spp.** The diagram shows the developmental stages of the parasite in the human and mosquito host. Figure adopted from Menard *et al*, 2013[9].

## **1.2. Epidemiology of *P. falciparum* malaria: geographical distribution, clinical features and risk factors**

*P. falciparum* infections occur primarily within the tropics and are limited to sub-Saharan Africa, South-East Asia, Latin America and the Middle East. In these regions however, the transmission intensity is not uniform and these differences influence the epidemiology of the disease [10]. Malaria transmission can vary greatly between regions and can occur throughout the year (perennial) or be highly seasonal with clear high and low transmission seasons occurring within a single calendar year. Various combinations or mixtures of these patterns have also been reported. The burden of malaria or its transmission intensity can be estimated using several indices such as the parasite prevalence in the 2-10 year olds ( $PfPP_{2-10}$ ) or the entomological inoculation rate (EIR), which is considered the gold standard [10]. The EIR estimates the number of infectious bites experienced per person per unit time. A second and easily obtainable metric is the  $PfPP_{2-10}$ , which calculates the proportion of 2-10 year olds within a particular region that have detectable circulating parasites. Based on this metric, geographical regions can be classified as being hypoendemic, mesoendemic, hyperendemic or holoendemic if the  $PfPP_{2-10}$  was less than 10%, 10-50%, 50-75% or greater than 75% respectively [10]. The epidemiology of clinical disease varies considerably between regions with varied transmission intensities.

### **1.2.1. Clinical features of *P. falciparum* disease**

The clinical progression and outcome of malaria can be influenced by multiple factors, including genetic/inheritable traits, naturally acquired immunity, environmental factors and variation in parasite virulence [11]. In non-immune individuals, infection with *P. falciparum* is almost always symptomatic even at very low parasite densities. Patients often present with intermittent fever and non-specific symptoms such as headaches, malaise, abdominal pain, muscle aches and diarrhoea [12]. If prompt diagnosis and treatment is not received, the disease can progress to a more severe and life-threatening complex syndrome that affects multiple organs. The symptoms in these severe cases include impaired consciousness (including

unrousable coma), shock, respiratory distress, body convulsions, hypoxia (decreased oxygen supply to the tissues), hypovolemia (decreased blood volume circulating in the body), hypoglycaemia (low blood glucose levels) and tachypnoea (abnormally rapid breathing) [13]. The symptoms in severe cases of malaria in non-immune adults and children are similar with the exception that adults are more likely to present with jaundice and acute renal or hepatic failure in addition to the symptoms listed above [12]. In children, three main syndromes can be observed during the course of severe disease that include severe malarial anaemia, respiratory distress and cerebral malaria, which share some overlapping symptoms. These syndromes are associated with an increased risk of death with an estimated 1%, 15% or 18% of children dying if they develop severe anaemia, respiratory distress or cerebral malaria respectively [14]. The recommended treatment guidelines for severe malaria include either the immediate delivery of intravenous doses of artesunate or intramuscular treatment with artemether. After 24 hours, if the patient is able to tolerate oral medication, the full course of an Artemisinin-based combination therapy (ACT) should be provided [12].

### ***1.2.2. Factors that influence the progression of disease***

The burden and severity of disease due to malaria is affected by multiple factors that can be classified as either being related to the geographical/social setting in which transmission occurs or in inherent properties of either the parasite or the host [14].

#### ***1.2.2.1. Geographical and social related factors***

The incidence profiles of severe disease and death due to malaria vary by age depending on the transmission intensity [15]. In areas of hypoendemic transmission, malaria infection and disease occur at low frequencies and both children and adults are at risk of severe complications and death. In contrast, in areas that are classified as being hyperendemic or holoendemic for malaria transmission, a distinct age-pattern is observed with severe clinical disease and death occurring primarily in young children while this risk of severe disease and death declines in older children and rarely occurs in adults [16]. In addition, the inability to access prompt diagnosis

and treatment increases the burden and severity of disease. These can occur due to political instability as well as cultural and/or economic reasons in which adequate treatment and drugs are either unavailable, inaccessible or cannot be afforded [17].

#### *1.2.2.2. Host related factors*

All non-immune individuals such as travellers of any age are susceptible to the severe manifestations of malaria. In endemic regions however, naturally acquired immunity (NAI) results in a clear age-dependent susceptibility to disease, although the extent and effect of this immunity is influenced by the transmission intensity of a given region. Additional host-related factors can also influence the course of infection in malaria endemic regions. First, immuno-compromised individuals, such as those who have undergone splenectomy [18, 19] or have acquired HIV/AIDS are at an increased risk of severe malaria following infection compared to healthy adults [20, 21]. Secondly, despite acquiring effective immunity against both severe and mild cases of malaria, pregnant women may become infected with *P. falciparum* and experience anaemia despite a low peripheral parasite density, as the majority of parasites localise to the placenta (PAM). This is observed particularly during their first (primigravidae) and second pregnancies [22]. PAM is associated with increased risk of stillbirths, premature delivery, neonatal death and low infant birth weight [23]. Lastly, inheritable traits have been identified that are associated with reduced susceptibility to either clinical disease, high parasite densities or infections. These include genetic mutations that affect the structure, function or quantity of either haemoglobin, erythrocyte surface proteins or enzymes [24]. These inheritable traits are discussed in more detail in section 1.4.

#### *1.2.2.3. Parasite related factors*

The severity of disease in children and pregnant women has been associated with several parasite related phenotypes. These include the emergence and prevalence of circulating drug-resistant parasites within a population, differences in parasite multiplication rates, and the ability of parasites to cytoadhere to tissues, organs and other cells. In addition, the ability of infected cells to adhere to: a) multiple



uninfected erythrocytes, a phenotype referred to as rosetting, b) clumping of infected erythrocytes to platelets and c) binding to dendritic cells have been associated with disease severity [13, 14, 25, 26].

*Drug-resistant parasites:* The spread of chloroquine resistance in the mid 1900s resulted in an increase in the mortality and severity of disease due to malaria in sub-Saharan Africa. Mutations in the chloroquine resistance transporter (*PfCRT*), particularly in position 76 (K76T) alongside several other amino acid mutations resulted in resistant strains that spread rapidly throughout Africa and Southeast Asia [27]. This prompted a change in treatment guidelines and a renewed search for effective anti-malarial drugs. The WHO currently recommends the use of ACTs as the first line of treatment of uncomplicated malaria [12]. Unfortunately, resistance to artemisinin has also emerged in South-East Asia manifesting itself as delayed parasite clearance following ACT therapy [28]. Resistance is associated with mutations within a Kelch protein located on chromosome 13 (K13 propeller domains)[29]. At the moment, resistance to ACTs in Africa, measured by monitoring the rate of parasite clearance, has not been reported [30]. Nevertheless, there is urgent need to identify new drug targets before resistance spreads to sub-Saharan Africa overturning the gains made in reducing disease and death in the last one and a half decades.

*Adhesion of infected erythrocytes to various tissues, organs or cells:* A key feature of *P. falciparum* infection is the ability of the parasite to express its own proteins on the surface of infected erythrocytes [14]. These parasite-derived proteins facilitate the cytoadherence (binding of infected erythrocytes to endothelial cells) of late-stage parasites to the brain, heart, liver, lungs, kidney, subcutaneous tissues, blood vessels and placenta with dire consequences [25]. Microvasculature obstruction by the mass of sequestered infected erythrocytes results in reduced blood flow and consequently oxygen, to tissues. In addition, systemic or local production of pro-inflammatory cytokines due to sequestration contributes to the pathology observed in severe malaria [14, 25]. These organs vary in the type and quantity of host

receptors that can be bound by parasites. In addition, parasites vary in the receptor ligands that they express which influence the specificity and affinity of cytoadherence and consequently disease progression.

The protein *PfEMP1* is one of the widely studied parasite ligands that mediate this cytoadherence [31]. The diverse *var* gene family, consisting of 60 genes, encodes this highly variable protein that consists of an N-terminal segment (NTS), several duffy binding like (DBL) and cysteine rich inter-domain regions (CIDR) domains. A single infected erythrocyte will express a single *var* gene. *In vitro* studies demonstrated that in a clonal infection and in the absence of immunity, the majority of the infected erythrocytes (approximately 98%) would express a single *var* gene with the remaining infected cells switching to a different member of the gene family in the following cycle [32, 33]. However in natural human infections, the number of *var* genes expressed and the rate at which they switch *in vivo* is likely to depend on the level of pre-existing antibody responses as well as the host tissue to which the infected erythrocytes cytoadhere to [34]. The specific *var* gene expressed can influence disease outcomes. For example, the parasites responsible for PAM have unique cytoadherence properties characterised by low binding to CD36, a host receptor in the microvasculature (that is the target for many *PfEMP1* proteins), and a high binding affinity to CSA, a host receptor in the placental intervillous space. These parasites express a unique *PfEMP1* protein termed VAR2CSA [35] on the surface of infected erythrocytes. Similarly, differences in the ability of infected erythrocytes expressing different *var* genes, to bind to the host receptors ICAM-1 or CD36 are thought to be associated with cerebral malaria and other clinical phenotypes of severe malaria respectively [25, 36-38]. Specific *PfEMP1* subtypes have been shown to be associated with severe childhood malaria [36, 39], and these were subsequently shown to bind to the previously unknown host receptor called endothelial protein C receptor (EPCR) [40].

Rosetting and platelet-mediated clumping by infected erythrocytes can be observed *in vitro* in parasite isolates obtained from infected individuals. These phenotypes are

thought to be associated with parasite virulence and severe malaria [25]. Rosetting is thought to occur when *PfEMP1* ligands on iRBCs bind to uninfected cells via CR1, heparin-sulfate like molecules and the blood group A and B antigens. This ability has been shown to be associated with severe malaria in Africa [41-46], and the *PfEMP1* ligands encoded by group A *var* genes were shown to mediate this adhesion [25]. Platelet-mediated clumping on the other hand, is thought to be mediated by an unidentified parasite ligand that interacts with CD36, globular C1q receptors and P-selectin on platelets [25]. Platelet clumping has been reported in cerebral malaria patients [47] and the phenotype has been associated with severe malaria in Kenyan [48], Malawian [49] and Thai [50] children.

*Parasite multiplication rates (PMR):* Differences in the rate at which parasites multiply have been thought to be associated with parasite virulence and the course of infection. In Thailand, *in vitro* PMR was significantly higher in isolates obtained from severe cases compared to uncomplicated malaria cases [51]. These differences however, have not been observed in isolates from Kenya and Mali [52]. Technical differences in the assays did not account for the discrepancy in these two studies as data from Thai isolates confirmed the initial findings made in Thailand [52]. The differences in transmission intensity and the levels of acquired immunity between South-East Asia and sub-Saharan Africa may partly explain the differences observed. South-East Asia is classified as a region of hypoendemic (low) transmission levels with low levels of acquired immunity within the population. In contrast, transmission levels in sSA vary ranging from hypoendemic to holoendemic malaria transmission with higher levels of pre-existing immunity present in individuals living in the regions of higher transmission intensity. Parasites adapted to grow in individuals lacking immunity reach high parasite densities quickly resulting in a high PMR. In contrast, parasites adapted to grow in semi-immune individuals may have evolved different mechanisms of virulence that are aimed at avoiding the host's immune system to facilitate onward transmission and the fitness cost of this evolution may be observed as a reduced PMR.

Despite some understanding of the different factors that influence the course of infection leading to death, the exact mechanisms and sequence of events that lead to severe malaria are unknown. Nevertheless, there is an urgent need for effective control measures to prevent both infection and disease. The benefits of effective malaria control include the prevention of the severe manifestations of the disease and the long-term neurological sequelae [53], as well as having an impact on childhood morbidity and mortality due to other diseases such as bacterial infections whose prevalence has been directly linked to malaria [54].

### **1.3. Control strategies for *P. falciparum* malaria**

Huge gains have been made since 2000 in the control of *P. falciparum* malaria, as shown by the reduction in deaths and clinical disease. These gains can be partly attributed to multiple control strategies in malaria-endemic regions [1, 10]. These include vector control measures, prophylaxis treatment in highly susceptible individuals and improvements in the rapid diagnosis and treatment of malaria. These are discussed briefly below.

#### ***1.3.1. Vector control***

Vector control measures are aimed at reducing the interaction between the mosquito vector and the human host. These measures include the use of insecticide treated nets (ITN), indoor residual spraying (IRS) and the management of larval breeding grounds [1]. The WHO has recommended that all children 5 years and under as well as pregnant women should sleep under an ITN. The use of ITNs in children has been shown to result in a marked reduction in the incidence and mortality due to malaria in endemic regions and is estimated to account for 50% of the fall in parasite prevalence in 2-10 year old children in sub-Saharan Africa (sSA) [55]. However, these gains are at risk of reverting following reports of mosquito vectors developing resistance to the four classes of insecticides used for ITN and IRS. Single-nucleotide substitutions in the *kdr* gene in *Anopheles gambiae* are associated with resistance to pyrethroids, the insecticides exclusively used in ITNs. In addition, cross-resistance, where the acquisition of resistance to one drug results in resistance to other insecticides with similar modes of action has been reported causing concern for future vector control measures[10]. Despite these worrying trends, ITN use has still been reported to be effective even in regions reporting high levels of resistance to pyrethroids [56].

#### ***1.3.2. Chemoprevention***

Intermittent preventive treatment (IPT) in infants (IPTi), pregnant women (IPTp) and seasonal malaria chemoprevention (SMC) in children aged 3-59 months are control measures aimed at preventing clinical episodes of malaria in highly

susceptible populations. For the widely adopted IPTp strategy, two doses of Sulphadoxine-pyrimethamine (SP) were recommended for all pregnant women in their second trimester and were associated with a reduction in placental malaria, anaemia, neonatal mortality and low birth weight[57, 58]. IPTi using SP has been recommended for use in areas of high transmission intensity provided SP resistance has not compromised its efficacy. Its use has been associated with over 30% protective efficacy against clinical episodes of malaria and hospital admissions associated with malaria parasitaemia [59]. In areas with highly seasonal malaria, SMC treatment with amodiaquine and SP has been associated with up to 80% reduction in clinical malaria and anaemia [60, 61]. Despite the clear potential gains and their recommendation by WHO, chemoprevention, particularly IPTi and SMC have not been fully adopted in endemic regions. While IPTp is in use in over 20 countries in sSA, only 31% of eligible pregnant women received the full dose in 2015 [1]. One concern over the widespread implementation of either of the above prophylaxis measures in endemic regions is the emergence and spread of drug-resistant parasites that might make the control measure obsolete [62]. It is clear that the commitment by governments and policy makers to ensure the implementation of these policies in endemic region needs to be scaled up.

### ***1.3.3. Rapid diagnosis and treatment***

The prompt detection and treatment of malaria could facilitate the prevention of severe malaria and death in patients and reduce the time in which parasites can be transmitted to susceptible hosts. The WHO has recommended the confirmation of every suspected malaria case using either microscopy or a rapid diagnostic test (RDT). These RDTs offer the ability to provide rapid diagnosis within the community particularly where laboratories equipped with microscopes, electricity and skilled personnel are lacking. The most commonly used RDTs detect a single circulating parasite protein, HRP2. Despite their utility, there are now reports of circulating parasites that lack the HRP2/3 gene in Southeast Asia and East and Central Africa [63-67] that could potentially affect the sensitivity of these RDTs.

New tests that include the detection of non-HRP2 antigens are available to confirm false-negative RDT results and to ensure that no cases are overlooked [68].

ACTs are the current recommended first-line of treatment for uncomplicated malaria [12]. Resistance to artemisinin, one of the component drug in ACTs was first reported in South-East Asia where delayed parasite clearance was observed as discussed in section 1.2.2.3. Despite these findings, ACTs remained partially effective due to the function of the partner drug. However, high failure rates of clinical treatment in Cambodia have been reported against four different ACT regimens [69], representing a major challenge for control and elimination.

#### **1.3.4. Vaccines**

Vaccines have long been recognised to be one of the major contributors to public health due to their effect in reducing the morbidity and mortality caused by infectious diseases. Vaccinations function by priming the host immune response to make an adequate protective response on exposure to pathogens at a later date [70]. So far, a highly effective malaria vaccine has remained elusive. RTS, S/AS01 (marketed under the trade name Mosquirix) is the only malaria vaccine that has been evaluated in phase III studies, resulting in an overall efficacy of only 29% and 39% against severe malaria and clinical disease, respectively [71]. This efficacy however, waned over time to less than 0% over a 7-year follow-up period [72]. This suggested that over time, vaccinated individuals were at an increased risk of a clinical episode of malaria compared to non-vaccinated individuals probably due to the inefficient or inadequate acquisition of NAI in vaccinated individuals due to less exposure to the parasites. Although not highly efficacious, the RTS'S vaccine received a positive scientific opinion from the European Medicines Agency (EMA) [73, 74] and WHO has recommended pilot studies to commence in 3-5 countries in sSA to test the feasibility, safety and impact on mortality of the vaccine alongside control measures already in use. New effective drugs, vaccines and insecticides are urgently needed to achieve sustained *P. falciparum* malaria control and ultimately elimination.

#### **1.4. Immunity to *P. falciparum* malaria**

Despite living in malaria-endemic regions, the susceptibility of individuals to both clinical disease and parasite infections vary. Epidemiological observations suggest that naturally acquired immunity to disease occurs with continuous exposure to malaria infections. Immunity to severe cases of non-cerebral malaria is thought to be acquired within the first two years of life [75]. Thereafter, children less than five years of age remain susceptible to clinical episodes of malaria, including cerebral malaria, but the incidence of these declines with age [76]. Older individuals seldom experience clinical episodes but remain susceptible to parasite infection. Despite these observations, differential susceptibility has further been observed even within highly susceptible age groups or populations that have been attributed to inherited traits such as Duffy-negativity, the sickle cell trait or G6PD-deficiency [24]. Genetically based and naturally acquired immunity to malaria are discussed in detail in section 1.4.1 and 1.4.2 respectively.

##### ***1.4.1. Genetically based resistance to malaria***

Humans and *Plasmodium* have co-evolved for hundreds of years, with *P. falciparum* DNA being detected in Egyptian mummies that were dated to between 500-2500BC (approximately 4000 years ago) [77]. This has resulted in the selection and/or fixation of inherited traits within the human population that confer protection against malaria. These genetic traits are highly prevalent in malaria endemic regions and include haemoglobinopathies, erythrocyte cell surface polymorphisms and immunogenic variants.

##### ***1.4.1.1. Haemoglobinopathies***

Genetic mutations or variants that result in an altered production or structure of haemoglobin have been shown to confer resistance to malaria [24, 78, 79]. Haemoglobin consists of 2- $\alpha$  and a 2- $\beta$  globin chains held as a tetramer and is found in the cytoplasm of erythrocytes. It is responsible for transport and distribution of oxygen within tissues. Haemoglobin disorders associated with protection from



malaria include: i) thalassemia (two types;  $\alpha$ -thalassemia or  $\beta$ -thalassemia), ii) sickle-cell trait (HbAS), iii) haemoglobin C (HbC) and iv) haemoglobin E (HbE). Although not a genetic disorder, the persistence of foetal haemoglobin (HbF) in infants has been thought to contribute to the protection against *P. falciparum* infection and disease observed in the first 6 months of life. While the exact mechanisms of protection are unclear, it is thought that the growth of parasites in HbF-containing erythrocytes compared to those with HbA is reduced, slowed or retarded [80-82].

$\alpha$ -Thalassemia is caused by a deletion of either one or both copies of the two  $\alpha$ -globin genes on chromosome 16 resulting in a decreased synthesis of the corresponding protein. The loss of one gene resulting in either the heterozygous  $\alpha$ -thalassemia ( $-\alpha/\alpha\alpha$ ) or homozygotes ( $-\alpha/-\alpha$ ) has been associated with protection from severe malaria compared to normal  $\alpha$ -globin phenotype ( $\alpha\alpha/\alpha\alpha$ ) [83, 84]. Similarly  $\beta$ -thalassemia is caused by mutations on the  $\beta$ -globin gene on chromosome 11 resulting in decreased synthesis of the corresponding protein. These traits occur in Africa and Asia as shown in Figure 1.2, with a prevalence of up to 48% and 17% for the  $-\alpha/\alpha\alpha$  and  $-\alpha/-\alpha$  genotype respectively, reported in Kenyan children [83]. The mechanisms by which these traits confer protection is not fully understood with *in vitro* observations suggesting that *P. falciparum* infected  $\alpha$ - and  $\beta$ -thalassemic erythrocytes bind higher levels of antibodies and were phagocytosed to a higher level when compared to normal erythrocytes [85-87]. In addition,  $\alpha$ -thalassemia has been associated with complement receptor 1 (CR1) deficiency, a receptor on erythrocytes involved in merozoite invasion [88] and associated with forming rosettes, a feature associated with severe malaria [89].

The sickle cell trait is caused by mutations in the  $\beta$ -globin gene that result in a change of the amino acid glutamic acid on position 6 to valine. The change results in structurally different haemoglobin molecules termed HbS, which polymerize and alter the shape of the erythrocyte forming a sickle shape. Homozygous forms of the

mutation, HbSS, results in sickle-cell disease and is fatal, while the heterozygous form (HbAS) is associated with protection from severe malaria [90]. The prevalence of the HbAS trait ranges from 10 to 40% across equatorial Africa and decreases to less than 2% in North Africa and South Africa where malaria transmission does not occur [91]. The proposed mechanisms by which sickle cell trait is protective include inhibition of parasite growth in HbAS erythrocytes, increased phagocytosis of sickle shaped parasite infected erythrocytes and reduced cytoadherence due to a reduction in the surface expression of the *Pf*EMP-1 protein [24].

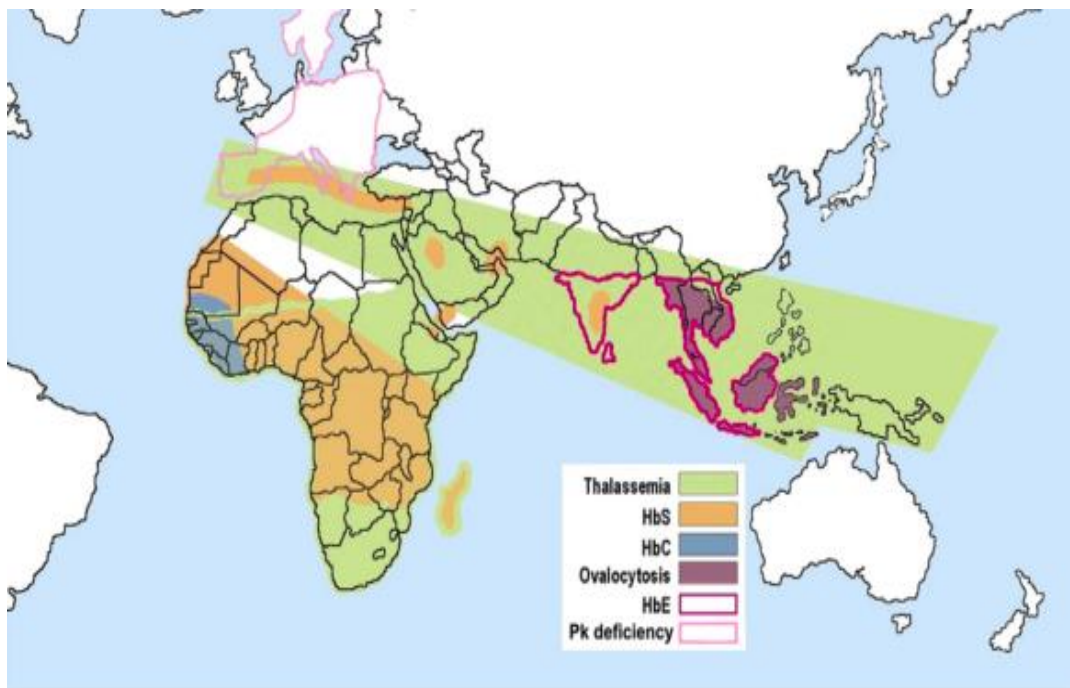
Additional variations in haemoglobin include HbC, caused by mutations on the  $\beta$ -globin gene that result in a change of the glutamic acid on position 6 to lysine, and HbE produced when the glutamic acid on position 26 is also replaced with a lysine residue. HbC is geographically restricted to West Africa while HbE occurs in South-East Asia (Figure 1.2). The prevalence of HbC is approximately 16% in West Africa and has been associated with fewer episodes of malaria and lower parasite burden in Malian children [92] although the mechanism by which the protection is conferred remains unknown. The prevalence of the HbE trait ranges from 5-10% in South-East Asia however currently, there is no evidence of protection conferred against malaria [93].

#### *1.4.1.2. Polymorphisms affecting erythrocyte surface proteins*

*Plasmodium spp* undergo an essential phase of their life cycle within erythrocytes and genetic mutations that have altered the expression of erythrocyte membrane proteins have been associated with resistance to malaria infection. These mutations include Duffy negativity, Gerbich negativity, South-East Asian Ovalocytosis (SAO) and defects in CR1 expression.

Duffy-negativity (Fy(a-b-)) is a phenotype that describes the lack of expression of the erythrocyte surface protein Duffy antigen receptor for chemokines (DARC) that results from a point mutation in the gene's promoter region, which is 33 bases upstream from the initiation codon. The DARC was thought to be the obligatory

receptor for *P. vivax* Duffy binding protein (DBP) with its loss halting *P. vivax* invasion of erythrocytes. Duffy negativity has reached near fixation (frequency >98%) in West and Central Africa and also occurs in Papua New Guinea. This explains the lack of *P. vivax* transmission in West Africa. Individuals who are heterozygous have approximately 50% reduced expression of DARC on erythrocyte surfaces [94], which has been associated with reduced *P. vivax* infection of erythrocytes [95]. However, transmission of *P. vivax* to Duffy-negative individuals has been reported in Kenya, Ethiopia, Madagascar, the Brazilian Amazon, Cameroon and Angola suggesting that the parasite has evolved an alternative pathway for invasion [96, 97]. In line with this, a second invasion ligand has been recently identified in *P. vivax* called erythrocyte binding protein 2 (EBP2) that although shows a strong preference for Duffy-positive reticulocytes, minimal binding to Duffy-negative reticulocytes was observed [98]. It has yet to be established whether this weak interaction can facilitate invasion in Duffy-negative individuals.



**Figure 1.2: The geographical distribution of inheritable traits that influence malaria susceptibility.** The map of the geographical distribution of hemoglobinopathies, erythrocyte polymorphisms and enzyme deficiencies that have been associated with protection from malaria. Figure is adapted from Lopez *et al* 2010[24].

Gerbich-negative blood group (Ge<sup>-</sup>), which occurs due to a deletion of exon 3 in the gene that encodes the glycophorin C (GYPC $\Delta$ ex3) surface protein, occurs at a high frequency of 46% in Papua New Guinea [99]. *In vitro* studies have shown that the mutation confers protection by preventing erythrocyte invasion by parasites using the EBA-140 invasion ligand [99]. However prospective studies have not yet demonstrated a protective effect of Gerbich negativity on malaria [100, 101]. A second geographically restricted polymorphism is ovalocytosis that is caused by the deletion of 27 base pairs in the gene encoding the erythrocyte membrane protein Band 3 on chromosome 17. Only the heterozygous form has been documented. It is prevalent in South-East Asia, causes no obvious clinical symptoms and is associated with protection from cerebral malaria [102]. Studies *in vitro* have suggested that the rigid ovalocytic erythrocytes are resistant to invasion by *Plasmodium* spp [103, 104].

Lastly, the level of surface expression of the CR1 protein on erythrocytes has been proposed to play a role in resistance to clinical malaria. The 190-280 KDa surface protein is the receptor for the parasite ligand RH4 on merozoites [88] and is also thought to mediate rosetting, a phenomenon associated with severe malaria in which late-stage infected erythrocytes bind to multiple uninfected red blood cells *in vitro* [105, 106]. The number of CR1 molecules per erythrocyte varies from between 50-1500, with marked reductions in rosetting observed in erythrocytes with less than 100 CR1 molecules. Reduced numbers of CR1 on erythrocytes is common in malaria-endemic regions with over 80% of Papua New Guinean population having less than 200 CR1 molecules per erythrocyte [107, 108]. The level of CR1 expression is influenced by both genetic factors and age, as was demonstrated recently in Kenyan children [89]. CR1 levels are genetically influenced by various mutations in the Knops blood group antigens, while the variations in the size of the antigen occur due to deletions or duplications in the long homology repeat units of the gene during crossover [109]. Low CR1 expression has been associated with protection from severe malaria in Papua New Guinea [107] and India [108].

A recent large genome-wide study involving over 11,000 African children identified a new cluster of genes that were associated with 33% protection from severe malaria [110]. Interestingly, all mutations identified to be protective were either next to or were themselves genes that play a key role in erythrocytes, such as those encoding the glycophorin proteins on erythrocyte surface that are known host receptors for parasite ligands.

#### *1.4.1.3. Polymorphisms affecting enzyme activity*

Mutations that have altered the function of several enzymes within red blood cells have also been associated with protection from malaria [24]. The reduced enzyme activity of the glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) has been associated with resistance to malaria. The G6PD enzyme catalyses the conversion of NADP to its reduced form NADPH, which in turn protects erythrocytes from oxidative damage. Normal G6PD activity is reduced in infected erythrocytes compared to uninfected ones [111]. Many different mutations have been described in multiple populations that result in reduced activity resulting in G6PD deficiency. *In vitro* studies have suggested that infected G6PD-deficient erythrocytes are more susceptible to phagocytosis particularly at the ring stages, which may reflect the protective associations observed in endemic regions [112]. The PK enzyme catalyses an irreversible reaction in the glycolysis cycle required for ATP synthesis in cells including erythrocytes. PK-deficient human erythrocytes have demonstrated resistance to *in vitro* infection with *P. falciparum* parasites and similar to observations with G6PD-deficient infected erythrocytes, increased phagocytosis has been observed in ring-infected PK-deficient erythrocytes [113].

#### *1.4.2. Innate immunity to malaria*

Insights into the role of innate immunity in malaria has been obtained from observations made in rodent malaria infections, *in vivo* evaluation of experimental *P. falciparum* infections in naïve individuals and *in vitro* evaluation of the effect of co-incubating parasitized erythrocytes with PBMCs from naïve donors. These innate

responses are thought to play a role in controlling the initial wave of parasitaemia prior to the development of adaptive immune responses [114]. In addition, immune cells that form part of the innate immune response act as bridge linking the innate response to adaptive responses. For instance, antigen-presenting cells such as dendritic cells are required to activate CD4<sup>+</sup> T-cells that subsequently produce IFN $\gamma$  as well as mediate antibody class switching to form the protective cytophillic antibodies observed in adaptive immune responses. The arms of the innate immune system that have been studied in the context of *P. falciparum* malaria include macrophages, NKT cells,  $\gamma\delta$  T-cells, dendritic cells and natural killer cells [114].

These innate immune cells have pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) on microbes resulting in activation of several downstream pathways that result in pathogen clearance [115]. *P. falciparum* PAMPs that have been identified include GPI anchors, haemozoin and immunostimulatory nucleic acid motifs. These molecules have been shown to interact with PRRs such as Toll-like receptors (TLRs) resulting in the induction of pro-inflammatory responses. For example, monocytes obtained from naïve individuals have been shown to be activated by haemozoin resulting in the production of TNF and IL-1 $\beta$  [116]. These pro-inflammatory responses such as IFN $\gamma$  function by activating effector function in innate cells. For example, monocytes have been shown to phagocytose infected erythrocytes in the absence of malaria specific antibodies. Scavenger receptors on monocytes including CD36 are thought to mediate part of this antibody-independent phagocytosis [117].

Unfortunately, the excessive induction of pro-inflammatory responses characterised by cytokines storms observed during acute malaria episodes partly contribute to the pathophysiological events observed in *P. falciparum* malaria. For examples, this cytokine storm is thought to suppress erythropoiesis contributing to the severe anaemia symptoms observed in *P. falciparum* infections [118].

#### ***1.4.3. Naturally acquired immunity (NAI) to malaria***

Historical observations and epidemiological studies in malaria endemic regions suggest that immunity to malaria is acquired following continuous exposure to the parasite. This hypothesis was based on the observations of the differences in the susceptibility to clinical episodes in adult travellers from non-endemic regions (non-exposed) and the adults resident in endemic regions [16]. Thereafter, epidemiological surveys in endemic regions demonstrated differences in the occurrence and frequency of disease as well as parasite densities between different age groups within the same geographical location and between different regions of endemicity [11]. In areas of high endemicity, a distinct age-dependent pattern in the frequency of clinical disease and in the density of parasites following infection was evident. Clinical disease rarely occurred in older children and adults and parasite densities similarly declined with age [119]. In contrast to this, the frequency of clinical episodes is uniformly distributed between age groups with older children and adults remaining susceptible to clinical episodes of malaria and high parasite densities in areas of low transmission intensity such as South-East Asia. NAI is generally thought to be: i) acquired at a rate that varies depending on the transmission intensity, ii) *Plasmodium* species specific, iii) achieved slowly and iv) requires continuous exposure (it is lost to some degree upon the cessation of exposure).

Firstly, as described in section 1.2.2.1, transmission intensity which determines the degree of exposure experienced by each individual, has an important influence on the rate at which immunity is acquired [15]. In hypoendemic regions, both adults and children remain susceptible to clinical malaria in contrast to the observations made in hyper- and holoendemic regions. In holoendemic regions, young children and infants (less than 2 years) are susceptible to high parasite densities and severe clinical malaria. They appear to acquire immunity to severe malaria and its complications following very few infections [75]. Surprisingly, immunity to cerebral malaria seems to be acquired later than resistance to other severe disease syndromes, as slightly older children (2-4 years) remain susceptible to CM [15]. In

addition, in this young age group (under 5 years), high parasite densities do not always lead to clinical manifestations, and the specific mechanisms at play that allow tolerance to high parasite densities are not fully understood. Nevertheless, with exposure, older children become less susceptible to mild malaria and high parasite densities and adults rarely show any symptoms. Secondly, studies in the 1920s using “malaria therapy” as treatment for neurosyphilis as well as epidemiological observations in endemic regions where both *P. falciparum* and *P. vivax* transmission occurred, established that the immunity acquired was species specific [120, 121]. NAI developed against one species was ineffective against the other.

Thirdly, antigenic variation that was first demonstrated in 1965 in *P. knowlesi* [122], may partly explain the slow acquisition of NAI to malaria. In this study, a clonal infection resulted in waves of parasites that were antigenically distinct resulting in the generation of variant specific antibody responses [122]. This provided a possible explanation for the slow acquisition of immunity observed in humans. In addition, random mutations and recombination over the asexual and sexual stage of the life cycle generates diversity in parasites. As a result, it is thought that repeated infections with diverse parasites were required in order to accumulate a broad range of isolate and variant specific antibody responses that eventually provided protection under natural conditions. Lastly, this immunity appears to be lost to some degree when exposure ceases. For example, adults who have emigrated from endemic regions and settled in non-endemic countries seemed to become susceptible to clinical disease on travelling back to endemic regions. However, when compared to naïve adults, these adults still experienced less severe disease and symptoms suggesting that the acquired immunity is not lost completely [123-126]. Despite the lack of clear understanding of the acquisition of immunity, identifying the targets of NAI, the immunological mechanisms by which it is achieved and maintained and the potential immune evasion strategies employed by the parasite remains central to vaccine development.



## **1.5. Immune effector mechanisms in NAI**

The parasite has a complex life cycle in which some phases are extracellular while others are intracellular and localize to different regions of the host including the skin, liver and blood. As a result, different immune mechanisms are thought to be important and include both the innate and adaptive immune system. One of the key mediators of NAI are antibodies that can be targeted at either the pre-erythrocytic or erythrocytic stages of the life cycle. The importance of antibodies was first demonstrated in seminal studies in which the passive transfer of immunoglobulins from adults resident in endemic regions was successfully used to treat children hospitalised with malaria [127-129], including those who lived in a region that was geographically distinct from the source of the IgG [129, 130]. This treatment resulted in a rapid drop in the parasite densities and a resolution of fever [127]. Identifying the targets of these protective immunoglobulins has been a high priority for the research community. Targets identified to date include proteins on the motile sporozoites prior to hepatocyte invasion, merozoites prior to erythrocyte invasion, parasite-derived proteins expressed on the surface of iRBCs and circulating gametocytes.

### ***1.5.1. Pre-erythrocytic immunity***

Both antibodies and T-cell immunity are induced by the pre-erythrocytic stages of the parasite [131]. Antibodies can be involved in blocking a) sporozoite motility from the dermis to the liver [132], b) invasion of hepatocytes by blocking receptor-ligand interactions [133] and c) interaction with monocytes and macrophages leading to the phagocytosis of sporozoites (Figure 1.3A) [134]. Once in the liver, infected hepatocytes can present parasite antigens on their MHC class I and II surface molecules. These will then induce CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses respectively (Figure 1.3B) [135].

### ***1.5.2. Erythrocytic immunity***

Antibodies are thought to be the main mediators of immunity during the asexual stages of the life cycle [136]. Erythrocytes lack MHC class I and II molecules and

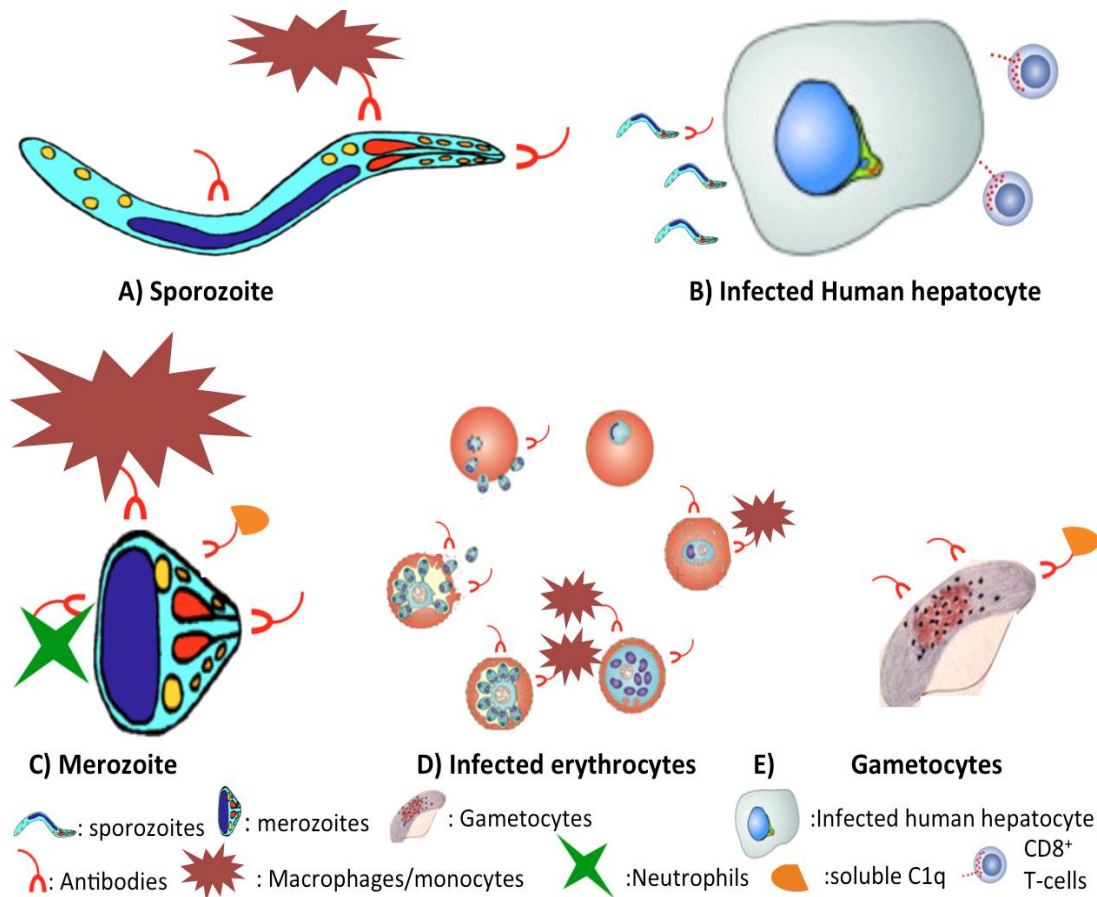
are therefore unable to induce T-cell responses that would directly eliminate iRBCs. Antibodies targeted against the merozoite can function in multiple ways (Figure 1.3C) that include: i) prevention of invasion and growth of the parasite in erythrocytes, ii) prevention of egress from schizonts [137], iii) interaction with soluble complement factors resulting in merozoite lysis [138] and iv) interaction with leucocytes such as monocytes/macrophages [139] and neutrophils [140, 141] leading to direct phagocytosis or release of toxic substances such as reactive oxygen species that result in parasite death (Figure 1.3C). Antibodies targeted at parasite-derived proteins on infected erythrocytes function by preventing cytoadherence, interacting with macrophages leading to phagocytosis and agglutinating iRBC that can be cleared by the spleen (Figure 1.3D) [34, 142]. Although CD8<sup>+</sup> T-cells are not involved during the erythrocytic stages, antigen-specific CD4<sup>+</sup> T helper cells are required for optimal antibody production by influencing the maturation and multiplication of antigen specific B-cells and thus the isotype of IgG produced [143, 144]. As a result, they can potentially influence the effectiveness of antibodies particularly those that interact with leucocytes via their cytophillic immunoglobulins.

### ***1.5.3. Immunity against sexual stages***

Gametocytes are also targets of natural-acquired immunoglobulins that are effective within the human and vector host. Gametocyte specific antibodies have been shown to interact with complement leading to gametocyte lysis within the human host (Figure 1.3E) [145]. In the mosquito vector, antibodies may function by a) prevention of gamete fusion and consequently zygote formation, b) induction of complement-lysis of gametes and ookinetes and c) prevention of ookinete motility, penetration of the midgut wall and formation of oocysts.

The majority of the proposed effector mechanisms described above have been obtained from studies using rodent malaria infections. Prospective cohort studies in humans have been used to identify targets of antibodies and immune effector mechanisms that are associated with protection from clinical disease [136]. For

example, antigens on the surface of or secreted by merozoites elicit antibody responses that were found to be associated with protection from clinical disease and high parasite densities in Kenyan [146] and Papua New Guinean [147] children. Similarly, antibody mediated effector mechanisms such as the phagocytosis of sporozoites [134], infected red blood cells [148] and merozoites [139, 149], antibody-mediated respiratory burst (ADRB) [140], antibody-dependent cellular inhibition (ADCI) [150] and antibody-dependent recruitment of complement (C1q-fixing) [138, 145] have been described in naturally exposed individuals. These effector mechanisms have each been shown to be associated with protective immunity. The specific parasite targets that elicit protective responses are the subjects of on going research. Additional insights into the targets and effector functions that are important can now be obtained from experimental human malaria challenge studies or naturally exposed populations [151, 152].



**Figure 1.3: Adaptive immune effector mechanisms in *P. falciparum* malaria.** An illustration of the adaptive immune responses (antibodies and T-cells responses) targeted at the motile sporozoite (A), infected hepatocyte (B), free merozoites (C), infected red blood cells (D) and gametocytes (E).

## 1.6. Immune evasion mechanisms

Despite the multiple immune-effector mechanisms elicited during natural infections, the parasite has developed elaborate evasion mechanisms that result in the development of clinical symptoms and the establishment of chronic infections. These mechanisms include the evasion of protective T-cell and antibody responses, avoidance of complement lysis, escape from clearance by the spleen and the occupation of host niches that may be protected from immune responses [153, 154].

### 1.6.1. Redundancy in erythrocyte invasion pathways

Invasion of erythrocytes by merozoites is a highly coordinated process that involves multiple proteins on the surface of the merozoite and secreted by merozoite organelles namely micronemes, rhoptries and dense granules. In rodent malaria, studies demonstrated that each merozoite derived from a single iRBC could express a different invasion ligand resulting in multiple erythrocyte invasion pathways (Figure 1.4A) [155]. In *P. falciparum*, two gene families important in this regard namely the reticulocyte-binding homologue (RH) and the erythrocyte-binding homologue (EBL) families encode a total of 12 proteins as shown in Table 1.1. Two of these are pseudogenes, meaning that there are at least ten ligand/receptor combinations that can theoretically be used for invasion. Targeting these parasite invasion ligands has been shown to inhibit invasion *in vitro* in some strains, but genetic studies have shown that many of the ligands are redundant. This presumably facilitates the escape from pre-existing antibody responses *in vivo* [156]. To add to this complexity, some of these targets such as EBA-175 are polymorphic, eliciting strain specific responses.

**Table 1.1: Table shows the *P. falciparum* invasion ligands and their host receptors**

Parasite ligand	Host Receptor	Notes
Reticulocyte-binding homologue (Rh) family		
RH1	Unknown receptor	Redundant
RH2a	Unknown receptor	Redundant
RH2b	Unknown receptor	Redundant
RH3	-	Pseudogene
RH4	Complement receptor 1[88]	
RH5	Basigin [157]	Essential pathway
Erythrocyte-binding homologue (EBL) family		
EBA140 (BAEBL)	Glycophorin C [158]	Redundant
EBA165	-	Pseudogene
EBA175	Glycophorin A [159]	Redundant
EBA181 (JESEBL)	Unknown receptor	Redundant
MAEBL	Unknown receptor	Possibly involved in sporozoite invasion
EBL1	Glycophorin B [160]	Pseudogene in some strains

### ***1.6.2. Antigenic polymorphism***

Genetic polymorphisms at specific gene loci result in antigenically distinct variants of the protein in different parasite strains or clones. This type of diversity has had an influence on both naturally acquired and vaccine induced immunity. Strain-specific immunity was first observed in the “malaria therapy” studies in which immunity was not always effective against heterologous challenge using different strains. Random genetic mutations can be generated in the parasite genome over every asexual mitotic division. Further diversity can be generated during the sexual reproduction within the mosquito where the two haploid gametocytes will result in four haploid sporozoite progeny [161]. As a result, for some antigens, many different haplotypes have been observed (Figure 1.4C). In some cases, these different haplotypes are able to abolish recognition by antibody responses leading to the selection of these parasites within the population. Antibody responses to specific

antigens have been grouped according the strain specific responses they elicit (serogroups). For instance, the merozoite surface protein 2 (MSP2) is largely divided into 2 serogroups namely group A (3D7-like) and group B (K1-like) whose amino acids can differ by up to 36% between serogroups [162]. Allele-specific naturally acquired antibody responses have also been reported to merozoite antigens such as AMA1 [163, 164], MSP-1 [165, 166], -2 [167-169] and -3 [170] in many populations including Kenya, Gambia, Mali and Papua New Guinea. These strain-specific responses are also induced following vaccination and are thought to have contributed to the low vaccine efficacy reported for the RTS'S [171], AMA1 [172] and MSP-2 [173] vaccines. In addition, some *P. falciparum* proteins contain blocks of highly repetitive protein sequences that can be present in several proteins. The number of the repeats present within an antigen confers a second source of polymorphism and is often highly immunogenic as illustrated with the circumsporozoite protein [174]. These highly immunogenic regions may act as a 'smoke screen' by diverting antibody responses from targeting sections of a protein that would elicit protective responses. Proteins with highly repetitive sequences can also stimulate B cells independently of T-cell help resulting in limited B-cell memory against these antigens [175].

### **1.6.3. Antigenic variation**

Antigenic variation was first demonstrated in *P. knowlesi* chronic infections [122] where they showed that subsequent waves of infection by the same parasite were antigenically distinct at the late stages of the erythrocytic life cycle. In *P. falciparum*, antigenic variation refers to changes in the antigenic phenotype that occurs due to the regulated expression of members of a gene family in a clonal infection, during the natural course of an infection. Since then, the large and extremely diverse *var* gene family has been described that encodes the *Pf*EMP1 protein implicated in antigenic variation [34, 176]. Additional gene families implicated encode the RIFINS, STEVORS, SURFINS and *Pf*MC-2TM proteins. These proteins on the surface of iRBCs are collectively termed as variant surface antigens (VSAs) and are targets of NAI eliciting variant-specific antibody

responses. The *P. falciparum* var gene family contains over 60 genes distributed across the 14 chromosomes whose expression is tightly regulated. Only one *PfEMP1* type is expressed on a single iRBC and this can change in the following cycle creating a variant that is antigenically different and thus evade pre-existing antibody responses (Figure 1.4B). This type of antigenic escape coupled to antigenic polymorphisms within these 60 genes makes *PfEMP1* a highly diverse protein that induces variant specific responses. In addition, *PfEMP1* also contributes to parasite evasion through other mechanisms such as cytoadherence which is thought to facilitate avoidance of splenic clearance of iRBCs [34].

#### ***1.6.4. Occupation of host niches protected from immune responses***

Parasite development within various cells in the human host has in itself provided an immune evasion mechanism. For example, liver schizonts release merozoite into vesicles called merosomes whose membranes are derived from the host cell. These merosomes are released to the circulatory system and parasite specific proteins are shielded temporarily from antibody effector mechanisms. Development within erythrocytes is another example that affords the parasite protection from both T-cell immunity and complement-mediated lysis. Erythrocyte surface proteins such as CD59, CD55 (DAF), CD35 (CR1) and CD46 (MCP) are complement regulatory proteins that prevent the formation of the membrane attack complex and consequently lysis of red blood cell [177, 178].

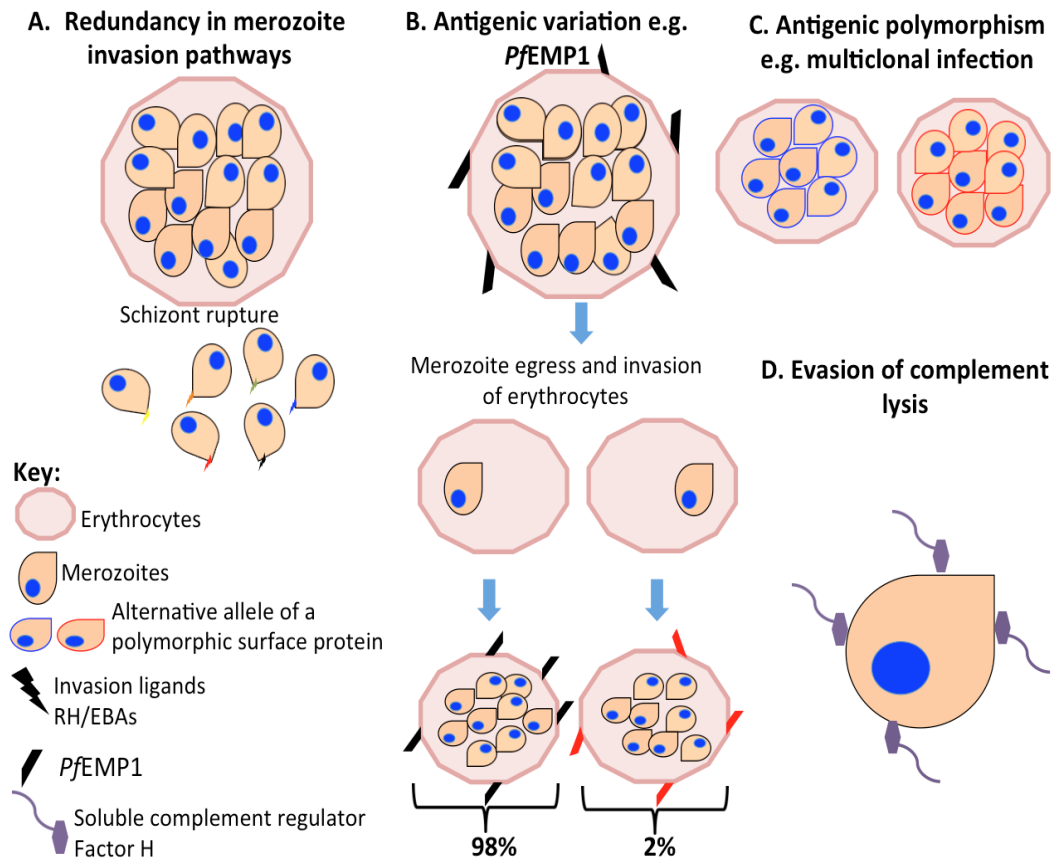
#### ***1.6.5. Evasion of the complement system***

Recently, specific surface proteins *Pf92* and *PfGAP50* on merozoites and gametes, respectively, were shown to bind to a soluble complement regulatory factor called factor H [179, 180]. Recruitment of factor H prevents activation of the complement pathway protecting the parasite from lysis (Figure 1.4D).

#### ***1.6.6. Epitope masking***

This is defined as the ability of non-parasite specific antibody responses to inhibit the binding of parasite specific antibodies to their epitopes. Recently, non-specific IgM have been shown to bind to *PfEMP1* variants such as VAR2CSA and appears

to protect iRBCs from IgG mediated phagocytosis[181]. Similarly, IgM binding to merozoite surface protein DBLMSP1 and 2 is thought to mask IgG binding. The consequence of this inhibition has not been fully established[182].



**Figure 1.4: Illustration of immune evasion mechanisms employed by *P. falciparum* blood stages.** A) Redundancy in erythrocyte invasion pathway facilitated by merozoites expressing alternative invasion ligands (represented as merozoites with apical ends of different colours), B) Antigenic variation in which the expression of the *PfEMP1* on the surface of infected erythrocytes switches within a clonal infection resulting in antigenically different late stages in the subsequent cycle (represented as infected erythrocytes with variant *PfEMP1* in black and red), C) Antigenic polymorphisms at a single locus resulting in the possibility of parasites that are diverse at this particular loci (represented as merozoites expressing different alleles of the same surface protein (blue and red merozoites) and D) evasion of the complement system by binding soluble regulatory protein factor H (purple molecule)



### **1.7. *P. falciparum* vaccine candidates in clinical trials**

Effective vaccines are considered the most powerful tool within the public health sector for the prevention or the control of an infectious disease for the following reasons [70]: i) they function by eliciting a protective immune response which provides an individual with immunity against disease following subsequent natural exposure to the pathogen, ii) in addition to resulting in a decrease in morbidity and mortality, vaccines can circumvent the economic and social burden due to hospitalization that can have an influence on the economic growth and productivity of an afflicted population and iii) they reduce the reliance on pharmacological drugs and/or pesticides (in vector-borne infectious diseases), which run the risk of acquiring resistance that would make them obsolete. While resistance to efficacious vaccines has not been reported [183], if the coverage of a vaccine against pathogenic strains is limited, non-vaccine strains can become prominent as has been observed with the PCV-7 vaccine [184] against *S. pneumoniae*.

The demonstration of both acquired and vaccine induced immunity to *P. falciparum* malaria has provided a strong rationale for vaccine development for malaria. Over 50 clinical trials have been conducted to evaluate vaccine candidates that have included whole parasite vaccines, sub-unit vaccines and combination of antigens in multi-subunit formulations. These have been derived from all three stages of the life cycle consisting of pre-erythrocytic, erythrocytic and transmission blocking candidates. There are strengths and weaknesses to targeting each stage. A completely effective pre-erythrocytic vaccine would halt parasite development at the liver stage, preventing disease altogether. However, a partially effective pre-erythrocytic vaccine would not eliminate severe disease, although it could reduce both the frequency and extent of the blood stage infection following exposure, and potentially reduce the severity of disease. This may occur by limiting the number of sporozoites that develop into mature liver-stage schizont and consequently the inoculum that initiates the blood-stage of the infection. Alternatively, some parasite proteins are present during the liver and blood stages of *P. falciparum* infection and

a vaccine based on such a target might confer both liver and blood-stage protective immune responses. By contrast, vaccines targeting the blood-stages are aimed only at reducing the severity of disease and target proteins involved in merozoite invasion of erythrocytes or intra-erythrocytic parasite development. Lastly, transmission-blocking vaccines target sexual stages preventing parasite development within the mosquito and subsequently transmission to the human host, but will have no effect on protection from disease severity in immunised individuals.

#### ***1.7.1. Whole sporozoite vaccines***

The first demonstration that experimental challenge with whole sporozoites conferred protection came from studies conducted in the early 1900s in which malaria was used to treat adult mental patients with neurosyphilis[121]. These early studies provided an opportunity to observe the clinical, parasitological and immune responses following a primary malarial infection and upon subsequent infection with either homologous or heterologous parasite strains[185]. A significant drop in the severity of symptoms, peak of parasitaemia and time to resolution of symptoms was observed in subsequent infections particularly with homologous strains and was more pronounced following repeated exposures[121]. Thereafter, in 1973, the first demonstration of sterile immunity following experimental challenge was observed using sporozoites from X-ray-irradiated mosquitoes[186, 187]. This immunity was subsequently shown to be dependent on the quantity of sporozoites used for immunization and conferred protection for a period of between 8-16 weeks[188]. Since then different sporozoite formulations have been or are currently under evaluation in clinical trials. These have included sporozoites that have been inactivated/attenuated through either radiation or genetically, or live non-attenuated sporozoites under prophylactic drug cover.

##### ***1.7.1.1. Radiation attenuated sporozoites (RAS)***

Sporozoites attenuated through radiation treatment and delivered via mosquito bites were used in the first experimental human challenge that demonstrated sterile

protection [187]. Over 1000 irradiated infected mosquitoes were required per individual, to induce a response that was protective in subsequent challenge with parasites[189]. This route of vaccine delivery was recognised as the major obstacle with this approach. Since then, Sanaria Inc., a biotechnology company has made improvements in obtaining aseptic purified cryopreserved radiation-attenuated sporozoites (*Pf*SPZ vaccine)[190]. This enabled the delivery of the vaccine directly overcoming the challenge of immunization via mosquito bites. This formulation has been tested widely in multiple clinical trials. Phase 1 trials showed limited protection when *Pf*SPZ was delivered via the intra-dermal (ID) or the sub-cutaneous (SC) route while intravenous (IV) delivery offered sterile protection to all the 6 healthy naïve adults challenged [191]. Since then the efficacy and duration of protection of *Pf*SPZ has been tested in malaria-naïve adults showing protection against both homologous and heterologous challenge over a 12-month and 3-week period respectively [192]. Encouragingly, *Pf*SPZ was safe and showed 49% efficacy against natural exposure in a randomised controlled trial involving healthy malaria-exposed adults in Mali over a 6-month follow-up period[193].

#### *1.7.1.2. Genetically attenuated sporozoites (GAS)*

A weakness of RAS is the precise dosing of radiation required for attenuation. As an alternative, genetic deletions of genes crucial for liver stage development have been used to generate GAS where the parasite life cycle arrests within the pre-erythrocytic stages. Following the observation of complete sterile protection in mouse models, the first human trial involved sporozoites in which the 6-cys p52 and p36 genes had been deleted (GASs p52<sup>-</sup>/p36<sup>-</sup>)[194]. These knockout parasites had been evaluated *in vitro* and using humanised mouse models and showed arrested liver stage development, as these genes are required for PVM formation. Encouragingly, of the six healthy malaria-naïve adults immunised with GASs p52<sup>-</sup>/p36<sup>-</sup> delivered via mosquito bite, five of the vaccinees remained free of parasitaemia for weeks after infection. In one individual, blood stage parasites were detected and clinical malaria experienced indicating substantial but incomplete attenuation [194]. Since then different knockout constructs have been tested for

complete attenuation in rodent and humanised mice models as well as *in vitro* hepatocyte infection. Complete attenuation has been recently reported for the GASs p52<sup>-</sup>/p36<sup>-</sup>/sap1<sup>-</sup> vaccine in which all the ten vaccinated adults remained parasite negative [195]. The effectiveness of the vaccine against challenge has yet to be reported.

#### *1.7.1.3. Sporozoite challenge under chemoprophylaxis cover (CPS)*

The chemoprophylaxis and sporozoite vaccination (CPS) strategy involves infection with live non-attenuated sporozoites under chloroquine cover that allows the completion of the pre-erythrocytic stage of the life-cycle releasing merozoites that initiate the blood stages. Chloroquine, which specifically targets the asexual stages, eliminates the blood-stage preventing clinical disease in vaccinated individuals. A phase 1 clinical trial involving 10 malaria-naïve adults vaccinated using the CPS (chloroquine) strategy via mosquito-bite showed complete sterile protection in all 10 vaccinees [196] that was effective up to 28 months after vaccination in 4 of the 6 vaccinated individuals[196]. These phase 1 results were replicated in additional studies involving malaria-naïve adults that involved escalating the dose of sporozoites used for vaccination[197] and in a CPS strategy in which chloroquine was replaced with mefloquine[198], a second licensed drug that targets asexual stages. As with RASs, the feasibility of vaccine delivery via mosquito bite was overcome using non-attenuated sporozoites from Sanaria. Inc. Complete sterile protection was recently observed in 9 malaria naïve individuals who were immunised with sporozoites via direct venous inoculation and challenged with sporozoites delivered by the same route[199].

While the results from clinical trials with whole sporozoite vaccines are encouraging, a number of questions remain unanswered. These include: 1) establishing the duration of the protection conferred, 2) effectiveness of the vaccination strategy in naturally exposed populations including pregnant women, immuno-compromised individuals and infants whose immune systems differ considerably from healthy adults, 3) effectiveness against heterogeneous parasites in

the context of natural exposure and lastly the feasibility of producing sufficient number of vaccine doses for use in malaria-endemic regions. The ability to scale production of any of these approaches to the numbers needed to economically immunize hundreds of millions of individuals in Africa remains a significant question.

### ***1.7.2. Whole blood-stage vaccines***

Studies in the 1940s demonstrated complete protection in monkeys and ducks immunised with adjuvanted *P. knowlesi* and *P. lophurae* infected erythrocytes respectively [200]. In subsequent studies in the 1970s, *P. falciparum* infected erythrocytes in complete Freund's adjuvant conferred protection against subsequent *P. falciparum* challenge in Aotus monkeys [201]. Only one clinical trial in humans using blood stage parasites has been conducted. Four healthy adults were treated with ultra low doses of infected cryopreserved erythrocytes under drug cover (atovaquone, proguanil and chloroquine). In three vaccinees, parasite DNA was not detected by PCR over a two-week period following challenge while a delayed patent infection was detected in one vaccinee [202].

Since then, the use of radiation, chemically or genetically attenuated blood stages as well as low doses of infected erythrocytes with different adjuvants have been and continue to be tested in mouse models [203]. Data from trials in humans have not been made public yet [203]. The production of whole blood stage vaccine faces several challenges. The use of human red blood cells to propagate parasites carries the risk of containing infectious agents that can be transmitted and therefore require rigorous screening. Secondly, vaccination may result in the generation of antibodies against erythrocyte surface antigens that may target self- antigens (auto-antibodies). As with whole sporozoite vaccines, the production, storage, and delivery of sufficient quantity of vaccines for all those at risk remains a challenge [203].

### ***1.7.3. Sub-unit vaccines***

In the early 1980s, the advent of recombinant DNA technology enabled researchers to clone and express *P. falciparum* proteins[204] that could then be evaluated as sub-unit vaccine candidates. As shown in Table 1.2, this technology enabled the identification of the majority of vaccine antigens that have so far been evaluated. According to the WHO rainbow tables [205], 22 parasite antigens have been or are currently being evaluated in clinical trials either as single antigens or in combination forming multi-subunit vaccines, 18 of which were identified and described before the *P. falciparum* genome[206] was published in 2002. This represents less than 0.5% of the proteins predicted to be encoded in the *P. falciparum* genome. The method of vaccine candidate discovery and the formulations under clinical trials are summarised in Table 1.2.

#### ***1.7.3.1. Pre-erythrocytic sub-unit vaccine candidates***

Seven antigens (CSP, TRAP, LSA-1, LSA-3, STARP, EXP-1 and *Pf*CelTOS) have been tested as pre-erythrocytic subunit vaccine candidates. These candidates are thought to function by eliciting antibody responses that target the motile sporozoite preventing hepatocyte invasion or elicit cellular responses that target infected hepatocytes. The most advanced candidate, CSP, is the parasite component of the RTS'S/AS01E vaccine that reduced the incidence of severe malaria and clinical disease by 29% and 39% respectively in phase III studies involving children aged 5-17 months in malaria-endemic regions [71, 207]. Pilot studies to test the feasibility, safety and the impact on mortality of the vaccine are set to begin in 2018 in five sub-Saharan African countries [3].

A second candidate, TRAP, has primarily been evaluated as part of a multi-component vaccine termed ME-TRAP that consists of multiple B-cell and T-cell epitopes (both CD4<sup>+</sup> and CD8<sup>+</sup>) fused to the TRAP protein. It is aimed at eliciting IFN $\gamma$  responses to eliminate infected hepatocytes. Different formulations have been tested with initial regimens demonstrating no efficacy in field studies[208, 209]. An alternative formulation (ChAd63 ME-TRAP) has been tested demonstrating partial

protection from parasite infection in Kenyan[210] and Senegalese adults[211]. Safety and immunogenicity trials with ChAd63 ME-TRAP have been conducted in children[212, 213], however their efficacy has yet to be reported. Similarly, *PfCelTOS*, a micronemal secreted protein is currently under evaluation in phase Ia trials. Protective efficacy was observed in *P. berghei* following both homologous and heterologous challenge with antibodies shown to inhibit sporozoite gliding and hepatocyte invasion[214]. The efficacy of this target in humans has yet to be reported. Lastly, multi-component pre-erythrocytic vaccines such as the FP9/MVA polyprotein that is composed of the TRAP, LSA-1, LSA-3, STARP, Pfs16 and EXP-1 showed no efficacy in phase I/IIa trials and was therefore discontinued from further evaluation[215]. A second multi-component DNA vaccine (polyepitope DNA EP1300) composed of multiple epitopes from CSP, TRAP; LSA-1 and EXP-1 underwent phase 1a studies in malaria naïve adults in 2015, but the results have not yet been published.

#### *1.7.3.2. Erythrocytic sub-unit vaccine candidates*

Eleven blood-stage antigens are or have been evaluated in humans as vaccine candidates. These include MSP-1, -2 and -3, AMA1, GLURP, SERA5, RESA and EBA-175 all of which were identified in the pre-genome era. Only three targets namely RH5, TEX1 (also known as P27) and VAR2CSA, were identified after the genome was published, emphasising how many potential antigen targets remain to be studied.

The most advanced blood stage vaccine is the GMZ2 that consists of the N-terminal of the GLURP protein fused to the C-terminal of MSP-3. Phase Ia and b studies in malaria naïve adults, semi-immune adults and children respectively, demonstrated safety and immunogenicity of the vaccine[216]. Recently however, a large multicentre phase 1b trial in children from Ghana, Burkina Faso and Uganda reported an overall vaccine efficacy of 14% against clinical episodes of malaria over a 6 month follow up period[217, 218]. The second blood-stage antigen MSP3 (181-276), has similarly demonstrated good safety and immunogenicity in both naïve and

semi-immune adults and children[219]. Preliminary reports on a phase 1b trial in Burkina Faso indicated a reduced malaria incidence in vaccinated groups[220].

Additional antigens that have been evaluated for efficacy in phase 2b studies and subsequently stopped, including EBA175 RII, FMP1/AS02A (MSP1<sub>42</sub>), combination B (RESA, MSP1 and MSP2) and FMP2.1/AS02A (AMA1) vaccines. Low vaccine efficacies (less than 10%) against clinical malaria have been reported for FMP2.1/AS02A [172], FMP1/AS02A [221] and combination B [222]. In the trials evaluating the FMP2.1/AS02A and combination B formulations, evidence of allele specific efficacy was observed, which was thought to contribute to the low overall efficacy [223]. Since these trials, blood-stage vaccine formulations under evaluation have been aimed at overcoming the challenge of antigenic polymorphisms. Several approaches have been trialled, including: i) inclusion of multiple alleles of a single antigen such as the *Pf*AMA1-DiCo, a diversity covering vaccine constructs that accounts for 97% of the amino acid variability observed in AMA1; ii) use of relatively conserved targets such as the SE36 (based on the N-terminal region of SERA-5) that showed a reduced incidence of malaria in vaccinees compared to control individuals [224]. Another conserved blood-stage vaccine ChAd63 RH5+/- MVA RH5, targets the essential and relatively conserved merozoite invasion ligand RH5; 3) targeting multiple antigens from both the pre-erythrocytic and blood-stages of the life cycle. These include GMZ2 vaccine and the NMRC-M3V-Ad-*Pf*CA that consists of a combination of CSP and AMA1. Lastly, a vaccine targeted at preventing placental malaria, PRIMVAC is undergoing phase Ia/b evaluation in healthy adults in France and Burkina Faso.

#### *1.7.3.3. Transmission blocking sub-unit vaccine candidate (TBV)*

In the 1970s, it was demonstrated in avian, rodent and monkey *Plasmodium* that immunisation of the host with sexual stages (gametocytes) generated antibodies that inhibited infectivity and sexual development within the mosquito[225, 226]. Subsequent studies identified *Pfs*230 and *Pfs*25 as targets of monoclonal antibodies[227, 228] that reduced mosquito infectivity and these targets are



currently under evaluation in Phase 1a studies. The most advanced TBV is the *Pfs25* proteins that localises to the surface of zygotes and ookinetes. Phase 1a studies have demonstrated that the Pfs25-EPA/Alhydrogel is safe and immunogenic in malaria naïve humans and induced antibodies with transmission blocking activity[229] as measured using an *in vitro* standard membrane-feeding assays (SMFA). Current TBVs face several challenges in their development and testing. First, the production of vaccines that are immunogenic and generate antibodies with transmission blocking activity has been difficult. Second, while *in vitro* SMFA can measure the function of vaccine-induced antibodies, this method is not practical for evaluating vaccine efficacy in the field where the key indicator would be a drop in overall transmission intensity [230].

**Table 1.2: Table giving a summary of 21 parasite antigens that have or are under evaluation in clinical trials as sub-unit vaccines; adopted from the WHO Rainbow Tables[205].**

Pre-genomic		
Pre-erythrocytic targets		
Antigen	Discovery	Vaccine constructs under clinical evaluation (past and present)
Circumsporozoite protein (CSP)	1982: cDNA library screening using monoclonal antibodies obtained from mice immunized with <i>P. knowlesi</i> sporozoites and immunoprecipitation of protein extracts from <i>P. knowlesi</i> sporozoites[231] [232].	Phase I-III: RTS, S/AS01E Phase II: RTS, S/AS02A, Phase I: CSVAC, R21/AS01B, R21/Matrix-M1 Phase I-II: NMRC-M3V-Ad-PfCA, DNA/MVA CSP Phase I: FP9 CSP + LSA-1 epitope/MVA CSP + LSA-1 epitope Phase I: HepB Core-Ag CSP-VLP Phase I-II: NMRC-MV-Ad-PfC, CSP long synthetic peptide Phase I-IIa: ChAd63/MVA (multivalent CS, ME-TRAP, AMA1) Phase I-IIa: RTS, S/AS01B + ChAd63 and MVA encoding ME-TRAP Phase I: polyepitope DNA EP1300, CSP. AMA1 virosomes
TRAP (Thrombospondin related anonymous protein) also known as PfSSP2 (sporozoite surface protein 2)	1988: Identified following the screening of the <i>P. falciparum</i> genome by Southern blot using a probe corresponding to the conserved region II of the circumsporozoite protein[233, 234].	Phase I-IIb: ChAd63/MVA ME-TRAP Phase I-II: FP9/MVA polyprotein Phase I: FP9 CSP + LSA-1 epitope/MVA CSP + LSA-1 epitope Phase I: DNA/MVA prime boost ME-TRAP Phase I: FP9 MVA prime boost ME-TRAP Phase I-IIa: ChAd63/MVA (multivalent CS, ME-TRAP, AMA1) Phase I-IIa: RTS, S/AS01B + ChAd63 and MVA encoding ME-TRAP Phase I: polyepitope DNA EP1300
Liver stage antigen 1 (LSA1)	1987: Screening of a genomic expression library using sera from individual exposed continuously to <i>P. falciparum</i> under chloroquine prophylaxis (sporozoite and liver-stage restricted sera)[235].	Phase I-IIb: ChAd63/MVA ME-TRAP Phase I-II: FMP011/AS01B Phase I-II: FMP011/AS02A Phase I-II: FP9/MVA polyprotein Phase I: polyepitope DNA EP1300
Liver stage antigen 3 (LSA3)	1990: Screening of a genomic expression library using sera from individual exposed continuously to <i>P. falciparum</i> under chloroquine prophylaxis (sporozoite and liver-stage restricted sera)[236]. 2007: screening cDNA library from erythrocytic stages using sera from malaria infected identifies LSA-3 as a liver and blood stage antigen[237].	Phase I-IIb: ChAd63/MVA ME-TRAP Phase I-II: FP9/MVA polyprotein Phase I-II: LSA-3 (inactive)
Sporozoite Threonine and Asparagine-Rich Protein (STARP)	1994: Screening of a <i>P. falciparum</i> expression library using sera from adults living in a malaria-endemic area	Phase I-IIb: ChAd63/MVA ME-TRAP Phase I-II: FP9/MVA polyprotein

	under long-term chloroquine prophylaxis (sporozoite and liver-stage restricted sera) [238]	
Pfs16	1991: Identified by screening a cDNA library generated from gametocyte RNA[239]. Rabbit polyclonal sera against the C-terminal region reacted with a 16KDa antigen from lysates obtained from gametocytes, gametes and sporozoites	Phase I-II: FP9/MVA polyprotein
Exported protein 1 (EXP-1)	1985: Screening of a cDNA expression library using sera from immune adults from Papua New Guinea[240].	Phase I-IIb: ChAd63/MVA ME-TRAP Phase I-II: FP9/MVA polyprotein Phase I: polyepitope DNA EP1300
<b>Erythrocytic targets</b>		
<b>Antigen</b>	<b>Discovery</b>	<b>Vaccine constructs under clinical evaluation (past and present)</b>
Merozoite surface protein 1 (MSP1)	1982: Immunoprecipitation of parasite lysate (schizont) using a monoclonal antibody that bound to the merozoite surface[241].	Phase I: FMP1/AS02, PfCP2.9 Phase I-II: FMP1/AS02A Phase I: MSP1-C1/AlOH + CpG Phase I-II: Combination B (RESA, MSP1, MSP2) Phase I: JAIVAC (MSP1-19/EBA175), FMP010/AS01B Phase I-II: ChAd63/MVA MSP1 Phase I: BSAM-2/Alhydrogel+CPG 7909, ChAd63 MSP1/MVA MSP1
Merozoite surface protein 2 (MSP2)	1985: Immunoprecipitation of parasite lysate (schizont) using monoclonal antibodies that bound to the surface of merozoites[242].	Phase I-II: MSP2-C1/ISA720 Phase I-II: Combination B (RESA, MSP1, MSP2)
Merozoite surface protein 3 (MSP3)	1994: Screening of a cDNA library using antibodies that inhibited parasite growth in the ADCI assay and bound to the surface of merozoites[243].	Phase I-II: GMZ2 Phase I-II: MSP3 (181-276)
Apical Membrane Antigen 1 (AMA1)	1982: Screening <i>P. knowlesi</i> parasite lysate (schizont) using monoclonal antibodies with growth inhibitory activity[244].	Phase I: pfAMA1-DiCo Phase I-II: NMRC-M3V-Ad-PfCA Phase I-IIa: ChAd63/MVA (multivalent CS, ME-TRAP, AMA1) Phase I: PfCP2.9, AMA1-FVO (25-545), AMA1-C1/ISA720 Phase I-II: FMP2.1/AS02A, AMA1-C1/Alhydrogel + CPG 7909 Phase I: BSAM-2/Alhydrogel+CPG 7909,CSP. AMA1 virosomes Phase I-II: FMP2.1/AS01B, ChAd63 AMA1/MVA AMA1 Phase I-II: ChAd63 AMA1/MVA AMA1 + alhydrogel/CPG7909
Erythrocyte Binding antigen-175 (EBA175)	1985: Intact erythrocytes were used to affinity purify parasite ligands from <i>P. falciparum</i> culture	Phase I-II: EBA175 RII Phase I: JAIVAC (MSP1-19/EBA175)

	supernatant[245].	
Ring-infected surface antigen (RESA)	1983: Screening of a cDNA expression library using immune sera that inhibited parasite growth <i>in vitro</i> [204, 246]	Phase I-II: Combination B (RESA, MSP1, MSP2)
Glutamate rich protein (GLURP)	1991: Screening of a <i>P. falciparum</i> expression library with human hyperimmune antibodies obtained following affinity purification using exoantigens (proteins released following schizont rupture). [247]	Phase I-II: GMZ2 Phase I: GLURP (85-213)
Serine repeat antigen (SERA5)	1987: Affinity purification from parasite lysates (schizonts) using a monoclonal antibody that was generated from exoantigens purified from culture supernatant[248].	Phase I: SE36
<b>Transmission blocking targets</b>		
<b>Antigen</b>	<b>Discovery</b>	<b>Vaccine constructs under clinical evaluation (past and present)</b>
Pfs230	1983: Immunoprecipitation of parasite protein from female gametes using monoclonal antibodies that interfered with transmission of gametes to mosquitoes[227].	Phase I: Pfs 230D1M-EPA/Alhydrogel
Pfs25	1985: Immunoprecipitation of parasite protein from macrogametes/zygotes using monoclonal antibodies that interfered with transmission of gametes to mosquitoes[228].	Phase I: Pfs 25 VLP Phase I: Pfs 25-EPA/Alhydrogel Phase I: ChAd63 Pfs25-IMMX313/MVA Pfs25- IMX313
<b>Post-genomic</b>		
<b>Pre-erythrocytic</b>		
<b>Antigen</b>	<b>Discovery</b>	<b>Vaccine constructs under clinical evaluation (past and present)</b>
Cell-traversal protein for ookinetes and sporozoites (CeTOS)	2006: Comparative analysis of expressed sequence tags (EST) databases of the salivary gland sporozoites and ookinetes to identify micronemal proteins[249].	Phase I: PfCeTOS FMP012
<b>Erythrocytic</b>		
<b>Antigen</b>	<b>Discovery</b>	<b>Vaccine constructs under clinical evaluation (past and present)</b>

Trophozoite exported protein 1 (TEX1)(P27A: PFF0165c)	2009: Bioinformatic screening for targets with $\alpha$ -helical coiled coil motifs, screening of synthetic peptides with immune sera and affinity-purified immunoglobulins inhibited parasite growth in the ADCI assay.	Phase I: P27A
VAR2CSA	2003: Compared the up-regulated gene in parasite isolates selected on adhesion to chondroitin sulphate A and isolates obtained from placental malaria (PAM) compared to isolates obtained from non-pregnant individuals[35].	Phase I: PRIMALVAC (PRIMVAC)
RH5	2002: Functional genomics analysis of the <i>P. falciparum</i> database identified the PfRH family, which shared homology and feature with both the <i>P. yoelii</i> and <i>P. vivax</i> reticulocyte binding homolog families.	Phase I: ChAd63 RH5 +/- MVA RH5

Green: pre-erythrocytic candidates. Purple: erythrocytic candidates. Blue: transmission blocking candidates

## 1.8. Antigen discovery and pre-clinical evaluation for vaccine development

Until the late 1990s, the conventional vaccination strategy had been based on the “isolate and purify the pathogen (or pathogen product) followed by an inactivation/attenuation/killing step then vaccinate susceptible hosts” approach. Edward Jenner had first applied a slightly different vaccination approach in 1796, where he used the wild-type cowpox (a less virulent poxvirus transmitted to humans from infected cattle) to vaccinate humans against the lethal smallpox virus [70, 250]. This approach has successfully generated licensed vaccines against viruses such as measles, rubella, mumps, varicella (chickenpox) and infectious bacteria such as *Mycoplasma tuberculosis* and *Clostridium tetani* (tetanus toxoid vaccine) [70]. In the “-omics” era, several high-throughput approaches have been employed to identify potential vaccine candidates. These include reverse vaccinology, comparative genomics, proteomics, transcriptomic, bioinformatic and computational modelling approaches [251, 252]. In the context of *P. falciparum* vaccine discovery, the majority of vaccine candidates under clinical evaluation (17 (81%)), were identified in the pre-genomic era (Table 1.2). These were identified primarily through the screening of genomic/cDNA expression libraries or testing the reactivity of parasite lysate material with monoclonal antibodies or sera of a defined specificity. In this era, antigen discovery was slow with only a single or few antigens reported in each publication.

The publishing of the *P. falciparum* genome in 2002 [206] revealed that the parasite had over 5600 proteins, a daunting number to examine individually. To overcome this challenge, several systematic approaches have been employed for antigen discovery in *P. falciparum*, including reverse vaccinology, population genetic studies and proteomic approaches. Each of these approaches however, has been greatly aided by publicly available “omic” datasets. These data sets include the comparative genomics [253], proteomics [254, 255] and transcriptional profiling [256-258] of *P. falciparum* parasites that have provided valuable information for consideration when selecting new malaria vaccine targets. Datasets that are

currently publicly available on PlasmoDB include the proteomic analyses of sporozoites, trophozoites, merozoites and gametocytes obtained from the *Pf3D7* and *NF54* strains [254, 255] and the transcriptional profiling of the *Pf3D7* and *PfHB3* strains throughout the intraerythrocytic developmental stages [259, 260], gametocytes and mosquito salivary gland sporozoites stages [260] to identify the stages in which candidates are expressed. In addition, large-scale genetic screens are now being carried out in the rodent *Plasmodium* model *P. berghei*, where essentiality data is now available for 50% of the genome, a high proportion of which have 1:1 orthologues in the *P. falciparum* genome [261].

### ***1.8.1. Reverse vaccinology***

Reverse vaccinology was pioneered by Rappuoli and colleagues [262] and involves the high-throughput *in silico* screening of the entire genome of a pathogen to identify genes that encode proteins with characteristics that were associated with immunity. Once targets were selected they could then be evaluated for immunogenicity and functional protection using various biochemical and cellular assays. The protein characteristics prioritised included the presence of signal peptide, transmembrane domains or GPI-anchors that suggest a protein may be localised on the surface of a cell and therefore accessible to antibodies. In *P. falciparum* target discovery, several research groups have coupled reverse vaccinology and the available “omics” datasets to select and validate candidates from the genome. For example both Fan *et al* [263] and Anand *et al* [264], used the following criteria to select targets from the *P. falciparum* genome: i) elevated mRNA transcription at the schizont stage (40-48 hpi) and ii) presence of a signal peptide or iii) one or more transmembrane domains or GPI-anchor and iv) have homologues present in different organisms of known function. One study subsequently identified 7 proteins namely MSP3.5, MSRP2, ETRAMP11.2, ETRAMP14.1, RALP1, StAR-related lipid transfer protein and a conserved membrane protein (PF3D7\_1459900) as immunogenic for the first time [263] while

the second study characterised the rhoptry associated adhesin (*Pf*RA) as a protein that translocates to the merozoite surface and interacts with erythrocytes [264].

Similarly, the genes encoding approximately a quarter of the hypothetical *P. falciparum* proteins were obtained from the genome and recombinantly expressed and printed on protein arrays, which were used for screening for antibody responses in naturally exposed individuals [265-268]. They achieved this by combining a rapid *in vitro* transcription/translation system to antibody screening by protein arrays. In these studies, a large number of *P. falciparum* predicted open-reading frames (ORFs) were recombinantly expressed using either the wheat germ cell-free protein synthesis systems (WGCFS) [269, 270] or a rapid transcription/translation system [265]. These recombinant protein fragments were printed on a microarray chip for analysis using sera from vaccinated individuals or those living in a malaria endemic region, with varying degrees of exposure and susceptibility to *P. falciparum* malaria [263, 265, 271]. These approaches identified novel targets of antibodies elicited following natural and experimental infection as well as those in individuals with sterile immunity to malaria following sporozoite vaccination [271, 272]. In addition, novel blood-stage targets of protective immunity and markers of exposure at an individual and community level have been evaluated [265, 267]. These studies continue to provide potential vaccine candidates that need further evaluation, although depend heavily on the quality of the expressed recombinant proteins and how well they mimic epitopes present in endogenous antigens.

### **1.8.2. Functional genomics**

Functional genomics refers to the comparisons of genes and genomes to identify proteins that share similarities suggesting shared or similar functions or interactions. In the context of *P. falciparum*, mining of the genome identified additional members of protein families [273-277], which shared homology and features with both the *P. yoelii* and *P. vivax* reticulocyte binding homolog families (functional genomics). One example is the RH family, which includes the reticulocyte binding proteins



(RBP) and the 235-kDa rhoptry protein initially described in *P. vivax* [278, 279] and *P. yoelii* [280, 281] respectively, and shown to be crucial in determining the red cell type invaded by merozoites. The *P. falciparum* members of this family are the RH genes, and the RH5 protein is a good example of a vaccine candidate identified through functional genomics [205]. Other members of the RH family continue to be evaluated in pre-clinical studies [282, 283]. Similarly, some members of the *P. falciparum* EBL family [284-288] were identified due to the similarities in the structure between these proteins and the Duffy-binding protein (DBP) of *P. vivax*. The characteristic features of these proteins include a cysteine-rich domain of approximately 35 kDa involved in host cell adhesion and first described in *P. vivax* [289] [289] and called the Duffy binding-like (DBL) domain. Members of the RH and EBL families continue to be evaluated as potential vaccine candidates, however the redundancy in the erythrocyte invasion pathways limits the utility of single targets with the exception of RH5 that is an essential invasion ligand [157].

### ***1.8.3. Population genetic analysis***

Analysis of the genetic differences between *P. falciparum* isolates has identified genes that are highly polymorphic in which several alleles of the gene occur at intermediate frequencies. These frequencies are higher than would be expected from random mutation and are thought to occur as a consequence of selection from the host's adaptive immune responses. Initial identification of targets under balancing selection were conducted using a panel of lab-adapted isolates and on a subset of genes [290]. These methods and findings were further validated using parasite isolates obtained from a Kenyan population [291]. The top hits identified from these studies namely MSPDBL1, MSPDBL2 and SURFIN 4.2 were evaluated in prospective cohort studies for immunogenicity and antibodies to MSPDBL2 were associated with protection from clinical episodes of malaria in Kenyan children [292]. These methods have been recently extended to a genome-wide scale using isolates from the Gambia identifying over 300 targets under balancing selection in the entire genome, raising the key challenge of down-selecting which targets to take

forward for pre-clinical evaluation. In addition, the vaccine efficacies of highly polymorphic antigens such as AMA1, MSP2 and CSP has been low, raising caveats about the use of similar polymorphic targets. If such targets are to be pursued, investment and continuous monitoring of the alleles in circulation in endemic regions would be required to guide in the development of a “diversity covering” vaccine.

#### ***1.8.4. Screening genomic or cDNA expression libraries***

Genomic or cDNA libraries refer to a collection of cloned DNA or cDNA fragments that have been generated by enzymatic digestion and transformed into host cells (usually *E.coli*) after ligation into expression vectors. The polypeptide gene products of the genomic or cDNA *E.coli* clones generated can then be screened for binding to antibodies. For instance, the immunodominant pre-erythrocytic sporozoite surface protein, CSP, was identified by screening a cDNA library constructed from mRNA obtained from *P. knowlesi* infected salivary glands [232] as well as screening sporozoite lysate material [231]. In these studies, they used hybridoma technology to generate mAbs from spleen cells obtained from BALB/c mice, which had been intravenously injected four times with *P. knowlesi* sporozoites. They then characterised these mAbs by testing for reactivity to the surface of sporozoites and testing the ability of mAbs to block infection on intravenous transfer of co-incubated sporozoites to rhesus monkeys. Thereafter, the protective monoclonal antibodies were used in immunoprecipitation experiments with sporozoite lysate material [231] as well as with the products of the cDNA expression library [232]. The specificity of this method was improved by analysing stage specific transcriptomes in which cDNA was generated from mRNA extracted from different stages of the life cycle. In addition, as shown in Table 1.2, the sera or monoclonal antibodies used were carefully selected. For example, the vaccine candidates LSA-1, LSA-3 and STARP were identified by screening cDNA libraries using sera from individuals who had been continuously exposed to *P. falciparum* but were under chloroquine prophylaxis that was effective against the blood stages of development

[235, 236, 238]. As a result, antibodies from these individuals were specific for the pre-erythrocytic phase of the life cycle. Recently, the same method was used to screen a blood-stage cDNA expression library using sera from 2 year olds that were either resistant or susceptible to *P. falciparum* parasitaemia and disease [137]. The antigen identified is now known as PfSEA-1. Antibodies to PfSEA-1 blocked parasite replication by preventing merozoite egress and Tanzanian children who had these antibodies did not experience severe malaria [137].

### **1.8.5. Proteomic approaches**

Proteomics refers to the large-scale identification and/or quantification of the set of proteins produced in the biological context either by whole organisms, organs or organelles. This approach has been used widely in the pre and post-genome era of vaccine discovery in *P. falciparum*. The two primary methods that have been utilised include immunoprecipitation techniques and the identification of parasite surface proteins on the premise that these are accessible to circulating antibodies.

Immunoprecipitation, also commonly referred to as “pull-down”, involves the precipitation of target antigens using monoclonal antibodies or immune sera. As shown in Table 1.2, it has been widely used in target identification. For example, monoclonal antibodies (mAbs) that had demonstrated *in vitro* parasite growth inhibitory activity [241, 242, 244] or interaction with monocytes leading to parasite death[243] were used in pull-down experiments to identify AMA1 [244], MSP-1 [241], -2 [242] and -3 [243] in the pre-genomic era. Similarly in the post-genomic era, the interacting partners of MSP1 and RhopH3 were identified in pull-down experiments using merozoite lysates and the respective anti-sera raised against recombinant MSP1 and RhopH3 [293]. These identified additional surface proteins such as MSP-6, -7 and -9 as well as the rhoptry proteins RhopH1, RAP-1 and RAP-3 as interaction partners of MSP-1. These are currently under pre-clinical evaluation as vaccine targets [147].

Membrane proteomics refers to the specific identification of proteins located within or associated with the surface of pathogens, which represent an ideal source of potential vaccine candidates as these proteins are exposed to the host immune system. A number of proteomic studies have been aimed at describing the merozoite sub proteomes, to identify merozoite anchored [294, 295], surface and secretory proteins [293, 296] as potential vaccine candidates. Detergent-resistant protein complexes as well as GPI-anchored membrane proteins obtained from *P. falciparum* schizonts were identified by mass-spectrometry. These identified new GPI-anchored and peripherally associated proteins such as *Pf92*, *Pf12*, *Pf38*, *Pf113* and *Pf41* and shown to be recognized by a pool of immune sera obtained from naturally exposed individuals [294]. Although none of these have reached clinical evaluation in humans, pre-clinical studies in humans [146, 147] and mice [297] continue to evaluate these targets [298]. Similarly, novel proteins on the surface of infected erythrocytes such as PIESP1 and PIESP2 [299] and recently on sporozoites from mosquito salivary glands [300, 301] have been identified using a capture and affinity purification technique. Data on their potential as vaccine targets have yet to be published.

### 1.9. Pre-clinical evaluation of potential vaccine candidates

Despite the availability of the genome for over 14 years, less than 1% of the parasite proteome has been or is currently being evaluated in clinical trials as vaccine candidates. The timeline between antigen discovery and clinical trials in humans has ranged from as short as 3 years to as long as 20 years [161]. Many proteins have been proposed to be potential vaccine candidates however data on pre-clinical evaluation is lacking and few targets have made it to the vaccine development pipeline. With the increasing number of targets identified, prioritizing antigens for vaccine development has become a challenge and remains a bottleneck for vaccine development.

Several pre-clinical evaluations should be considered to aid in prioritization. First, the responses generated following natural exposure to *P. falciparum* to these antigens should be evaluated in several cohorts to identify targets that are consistently associated with protection in varied transmission settings [302]. Secondly, additional *in vitro* assays have been developed that correlate with immunity to malaria [136] and the reliance on a single assay to measure effector functions may not provide a comprehensive evaluation of antibody targets [136]. Antigens that are able to elicit antibodies with multiple immune effector mechanisms could be prioritized and in theory should generate highly efficacious vaccines. Thirdly, the development of humanised mouse models and *P. falciparum*/*P. berghei* chimeric parasite lines may offer a rapid way of testing vaccine candidates for immunogenicity and efficacy prior to vaccine studies in humans. Lastly, the setting up of facilities that allow controlled human malaria infections (CHMI) in malaria-endemic regions such as Kenya [151] and Tanzania [303] has been an expensive venture. However, they offer the ability to measure the efficacy of vaccines rapidly, within weeks or months by comparing the prevention or reduction in parasite multiplication rates in vaccinees and control subjects. Thereafter, the long-term vaccine efficacy against heterologous *P. falciparum* infection as occurs in the natural setting will need to be evaluated in classic Phase III studies.

### 1.10. Aims and scope of the thesis

Seminal studies conducted over 60 years ago demonstrated that naturally acquired immunoglobulins conferred protection against clinical disease and high parasite densities. However, to date, only a small proportion of the parasite proteome has been evaluated in this regard. The overall aim of this study was to identify novel merozoite targets of protective immunity. Below, I outline the aims, rationale and a brief summary of the findings presented in the chapters of this thesis.

In **Chapter Two**, the aim was to identify merozoite proteins that are recognised by immunoglobulins from malaria immune adults. I used a 2-dimensional gel electrophoresis technique to resolve proteins extracted from *P. falciparum* merozoites and tested them for reactivity with a pool of immunoglobulins from both immune and non-exposed adults. I identified a total of 339 proteins that appeared to be reactogenic. Seventeen percent of these proteins have been previously shown to be targets of naturally acquired antibodies thus validating my approach. I found that a large proportion of the identified targets had not been previously evaluated in the context of naturally acquired immunity.

In **Chapter Three**, I set out to identify proteins that are localised on the surface of free merozoites. Although efforts have been made to identify merozoite membrane proteins, mining of PlasmoDB suggest the presence of many more unidentified proteins. I used complementary proteomic approaches that included cell surface trypsinization and biotinylation to identify surface proteins. I identified 374 putative merozoite surface proteins that included known GPI-anchored and peripherally associated proteins as well as membrane embedded multi-transmembrane proteins. Within this list include proteins that have been proposed to play roles in erythrocyte invasion and may be essential for *P. falciparum* survival. These may therefore represent attractive targets of naturally acquired immunity and consequently potential vaccine candidates.

**Chapter Four** focuses on the down-selection criteria I used to select a subset of 27 antigens for immunoprofiling. I generated recombinant proteins for 22 of these proteins for the first time, and found that 19/22 (86%) were recognised by a pool of hyper-immune sera from malaria-exposed individuals but not sera from non-malaria exposed individuals.

In **Chapter Five**, I measured antibody responses to eleven of the 22 recombinant antigens in a cohort of longitudinally monitored adults living in an area of hyperendemic *P. falciparum* transmission. I have i) confirmed that the recombinant proteins are recognised by sera from adults living in malaria endemic regions, ii) described the stability of antibody responses over a three-year period, iii) demonstrated that antibody responses to six antigens are significantly lower in adults who experienced a clinical episode compared to those who remained disease free and iv) established that the breadth of antibody responses to well-studied and newly identified immunogenic proteins are associated with protective immunity.

In **Chapter Six**, I examined the role of antibody-mediated functional activity in protection against disease and parasite densities in a malaria exposed adults. I focussed on three functional assays: opsonic-phagocytosis of merozoites (OPA), antibody-dependent respiratory burst (ADRB) and antibody-dependent recruitment of complement factor C1q (C1q-fixation). Little is known about the contribution of these effector mechanisms to NAI in adults, as previous studies have been limited to the development and validation of these immunological assays. Using the same cohort of adults in Chapter five, I showed that: i) opsonic-phagocytosis and the ability to fix complement was associated with protective immunity and ii) a strong positive correlation between antibody responses to the newly identified immunogenic antigens and protective effector function namely ADRB and C1q-fixation. This provides some evidence of the possible mechanisms by which antibody responses to these novel potential vaccine candidates function.

Finally, in **Chapter Seven**, I provide a summary of the findings generated in this thesis and recommendations for future studies.



## CHAPTER 2

### ***Plasmodium falciparum* merozoite antigen discovery using immunoprecipitation and bi-dimensional gel electrophoresis**

#### **2.0. Introduction**

Passive transfer studies conducted over 50 years ago established the role of naturally acquired antibodies in protection against *P. falciparum* malaria. In these studies, immunoglobulins from adults living in malaria-endemic regions were passively transferred to children admitted with a clinical diagnosis of malaria and high parasite densities. The result was a resolution of fever and a drop in parasite densities over a four day period to less than 1% of the initial densities [127]. These findings were replicated in subsequent studies where passive transfer using West-African adult human IgG was used successfully to treat children from East and West Africa [128, 130], as well as Thai patients [129]. Since then, the targets of these “protective” antibodies and the mechanisms by which they mediate protection have been under investigation.

Protective antibodies may be directed against a range of parasite proteins expressed during the multiple stages of the *P. falciparum* life cycle (see Chapter 1 section 1.1). These include pre-erythrocytic stage targets on the sporozoite or erythrocytic stage antigen targets present either on infected erythrocytes, merozoites or on gametocytes\*. All clinical symptoms experienced during *P. falciparum* infection are a consequence of the erythrocytic stage of the life cycle, which are initiated by the invasion of merozoites into erythrocytes. *P. falciparum* merozoites are the only extracellular parasite forms at the asexual stage of the life cycle and are therefore directly exposed to the host's blood stream and accessible to circulating antibodies [304, 305]. In addition, parasite derived antigens are exported onto the surface of

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\* Antibodies against gametocytes may serve as transmission blocking agents providing protection to endemic populations and not the individual per se.

infected erythrocytes in the late stages of the blood-stage of infection and are also major targets of naturally acquired antibodies [34, 142].

Identifying the targets of protective antibodies has remained a research priority since the discovery of their activity in passive transfer studies in humans. In the pre-genomic era [206], antigen discovery was primarily achieved using two complementary methods. The first method involved the construction of genomic or complementary DNA libraries expressed in *Escherichia coli* that could be screened using sera from infected humans, mice or monkeys. For example, the screening of a blood-stage cDNA library using sera from Papua New Guinean adults led to the identification of the immunodominant blood-stage S-antigen [204, 306]. Similarly, three blood-stage antigens that were localised on the surface of merozoites were identified by screening a cDNA library constructed from late-stage *P. falciparum* mRNA using sera from *Aotus* monkeys immune to *P. falciparum* infection [307]. The second approach was enabled by the discovery of hybridoma technology in 1975 [308], which permitted the *in vitro* production of monoclonal antibodies (mAbs) of defined specificities. These antibodies were then used to screen either cDNA libraries or parasite lysate material to identify target antigens. For example, a small number of known merozoite antigens that have in the past been considered leading vaccine candidates, such as AMA1 [244] and MSP-1 [241], -2 [242], -3 [243] (MSP- 1, 2, 3) relied on the screening and immunoprecipitation of parasite lysates with monoclonal antibodies of a defined specificity. Compared to the current techniques (described in Chapter 1 section 1.8), these methods led to the identification and characterisation of a relatively small number of antigens, over a long period of time.

More recently, the completion of the reference *P. falciparum* 3D7 genome, has allowed the use of more systematic and/or hypothesis driven approaches that have led to the rapid identification of multiple immune targets as has been discussed in section 1.8.1. Four main techniques have been employed to identify immunogenic merozoite antigens: i) parasite population genetic analyses (section 1.8.3), ii)

proteomic identification of membrane proteins (section 1.8.5), iii) literature review and selection of antigens of known function that may be immunogenic and iv) the use of various protein expression systems to generate large panels of parasite proteins for immunoscreening as has been discussed in Chapter 1 section 1.8. Although these approaches have increased the number of antigens under study as potential vaccine candidates, I identified a large number of merozoite proteins on PlasmoDB (<http://plasmodb.org>) that are likely to be accessible to circulating antibodies (described in detail in section 2.5.1). Of these potential targets, only approximately 20% had been evaluated for immunogenicity at the time this thesis was written, indicating that many more are yet to be discovered, evaluated and prioritised.

## **2.1. Rationale**

In this chapter, I sought to identify *P. falciparum* merozoite antigens that are immunogenic and therefore potential targets of protective antibodies. To do this, I used two complementary techniques: i) immunoprecipitation of *P. falciparum* merozoite lysates and ii) bi-dimensional gel electrophoresis coupled to western blots. For each technique, I used a pool of immunoglobulins from malaria-immune adults to identify potential protective antigens, while a pool of immunoglobulins from non-malaria exposed adults was used as a negative control.

### ***2.1.1. Immunoprecipitation***

Immunoprecipitation (IP) techniques have been successfully used to identify the targets of multiple monoclonal and human polyclonal antibodies against infectious agents such as *Vibrio cholerae* [309] and *Epstein-Barr* virus [310]. In the context of *P. falciparum*, IP techniques have mainly been used to “pull-down” targets in antigen discovery experiments and to identify protein-protein interacting partners. For example, monoclonal antibodies were used in pull-down experiments to identify targets such as AMA1, MSP1 and MSP3 in the pre-genomic era. Similarly, the

interacting partners of MSP1 and RhopH3 were identified in pull-down experiments using merozoite lysates and the respective anti-sera raised against recombinant MSP1 and RhopH3 [293]. While the above approaches have relied on either a monoclonal antibody or polyclonal sera against a single target, the extension of the technique to using polyclonal sera of broad specificity from malaria immune individuals could lead to the identification of multiple targets in a single experiment. For example, four novel *P. yoelii* blood-stage antigens were identified following pull-down experiments using pooled sera from mice that spontaneously cleared *P. yoelii* infection following challenge experiments [311]. The antigens that were identified in that study were subsequently shown to be partially protective in experimental immunization and challenge studies in mice [312]. I therefore sought to identify novel immunogenic *P. falciparum* merozoite targets by using a pool of purified immunoglobulins from malaria immune adults that is expected to have a wide range of specificities against merozoite expressed proteins.

### **2.1.2. Two-dimensional gel electrophoresis (2DE)**

The two-dimensional gel electrophoresis (2DE) technique has been widely used for many infectious agents to provide a snapshot of a pathogen's proteome at a specific time-point [313]. In this technique, complex mixtures of proteins are separated according to their isoelectric points (pI) in the first dimension of electrophoresis, followed by a subsequent separation according to their molecular weight by SDS gel electrophoresis in the second dimension. This can allow the resolution of approximately 2000 proteins to individual spots, which can be excised and identified by mass-spectrometry [314]. In malaria, it was first successfully used to identify *P. falciparum* merozoite and schizont specific proteins [315] and later used to identify extracellular secretory antigens at the erythrocytic stage of infection [316]. Coupling 2DE to serology has been employed in a few studies in the context of malaria. These have included i) identifying targets of protective antibodies against *P. knowlesi* schizont membrane proteins, using hyperimmune sera obtained from *P. knowlesi* infected rhesus monkeys (however, as the genome was not

available at that time, the identity of the immunoreactive targets remained unknown) [317], ii) identifying *P. falciparum* infected erythrocyte membrane proteins recognised by sera from briefly exposed individuals (travellers to malaria endemic region for a period of less than 6 months) [318] and iii) a serum factor,  $\alpha$ 2-macroglobulin, that binds to the *PfEMP1* variant HB3VAR06 [319]. One-dimensional gel electrophoresis (1DE) has been used in two other studies to measure reactivity of parasite lysate with sera from individuals with varying degrees of exposure and immunity to *P. falciparum* malaria [320, 321]. Differences in the pattern of recognition of parasite lysate material were clearly visible. However, a known limitation of this method, that separates proteins according to their molecular weight only, is that many proteins would be almost identical in size and therefore resolves similarly on a 1DE gel. Therefore assigning immunogenicity to a particular protein becomes a challenge.

I chose to use the 2DE technique coupled to western blotting to identify immunogenic merozoite antigens for the following reasons: i) 2DE technology is superior to 1D-SDS gel electrophoresis in the separation of complex protein mixtures into individual components, ii) a pool of purified immunoglobulins obtained from malaria immune adults expected to have a wide range of specificities, was available and would allow the identification of multiple immune targets and iii) the genome sequence of *P. falciparum* was publicly available and would aid in rapid target identification.

## 2.2. Overall Objectives

To identify immunogenic *P. falciparum* merozoite antigens using complementary proteomic approaches.

## 2.3. Specific Objectives

- To identify immunogenic *P. falciparum* merozoite antigens using immunoprecipitation techniques.
- To identify immunogenic *P. falciparum* merozoite antigens using two-dimensional gel electrophoresis techniques coupled to fluorescent immunoblotting.

## 2.4. Laboratory methods

### 2.4.1. *P. falciparum* merozoite purification and characterisation

The *P. falciparum* 3D7 strain (Pf3D7) and an isolate obtained from a child admitted to Kilifi County hospital in Kenya with cerebral malaria termed KIL9605, were maintained in *in vitro* culture through infection of human O<sup>+</sup> erythrocytes grown in standard parasite culture media (RPMI 1640 media supplemented with 30mM HEPES, 0.05mg/ml hypoxanthine, 0.025mg/ml gentamicin, 2mg/ml D-glucose, 3% Albumax II and 7.5% sodium bicarbonate) at between 8-10% parasitemia and at 5% haematocrit. A high parasitemia was required to obtain sufficient quantity of merozoites for downstream analysis. Parasites were synchronised repeatedly with 5% D-sorbitol treatment to obtain a culture where the majority of infected erythrocytes were in the ring-stages. This method relies on a *P. falciparum* encoded permeability pathway that is located on erythrocyte surfaces in the trophozoite and schizont-stages but absent in the ring-stages. This new permeability pathway (transporters) allows entry of D-sorbitol in the late stages resulting in cell lysis [322]. Synchronous parasites were returned to culture and allowed to mature to the late-stages. A second

synchronization step that uses a magnetic column (Miltenyi Biotec) was performed to isolate late trophozoite and early schizont parasites stages. This magnetic separation exploits the presence of haemozoin in the food vacuole (a by-product of haemoglobin breakdown) of late stage parasites (trophozoites and schizonts) [323]. Haemozoin contains iron in the ferric state ( $\text{Fe}^{3+}$ ) that has strong magnetic properties, and this allows separation of late-stages from the ring stages and uninfected erythrocytes obtaining a purified trophozoite/schizont culture (96-99% purity) [324]. Purified mature parasite stages were returned to culture and monitored until a majority were segmented (approximately 44 hours post invasion). Segmented schizonts were incubated with 2.5 $\mu\text{M}$  of Compound 2 (4-[7-[(dimethylamino) methyl]-2-(4-fluorophenyl) imidazo [1,2- $\alpha$ ] pyridine-3-yl] pyrimidin-2-amine) for 6 hours. This reagent prevents merozoite egress from schizonts by inhibiting protein kinase G (*Pf*PKG) and blocking the release of the contents of the micronemes and exonemes [325]. This inhibition is reversible upon removal of the reagent. Treated schizonts were pelleted by centrifugation, resuspended and incubated in fresh media to allow egress to occur over 30 minutes to 1 hour. The culture was then filtered through a 1.2 $\mu\text{m}$  Acrodisc filter to separate unruptured schizonts from released merozoites. Merozoites obtained were pelleted by centrifugation and used in downstream analyses.

#### ***2.4.2. SDS-PAGE analysis and Western blot***

Parasite material was resolved on a 4-12 % NuPAGE Bis-Tris gel after a 10 minute incubation at 70°C with 50mM dithiothreitol (NuPAGE Sample reducing agent) and lithium dodecyl sulphate, pH 8.4 with glycerol (NuPAGE LDS sample buffer). Pre-made gels were assembled on a X-cell Surelock mini-cell system and run using the MOPS SDS running buffer (50mM MOPS, 50mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7) for 90 minutes at 150 Volts. Proteins that resolved well on an SDS gel were either stained with Coomassie brilliant blue stain overnight or transferred onto polyvinylidene fluoride (PVDF) membrane for Western blotting. PVDF membranes were soaked in 100% methanol for 30 seconds before a single rinse in

water followed by incubation in transfer buffer (20X NuPAGE Transfer buffer). Pre-soaked sponge pads, blotting paper, resolved SDS gel, PVDF membranes, blotting paper and additional pre-soaked sponge pads were assembled in that order on the cathode core of the XCell II Blot module (Invitrogen) and sealed and assembled onto the X-cell Surelock mini-cell system. The blot module was filled with transfer buffer and outer chamber filled  $\frac{3}{4}$  way with distilled water and transfer done either at 30V for 1 hour at room temperature or overnight at 4°C at 10V. Following transfer, PVDF membranes were blocked in 5% non-fat milk/0.1% Tween20/PBS for 2 hours at room temperature before incubation with the respective primary antiserum at a 1:500 dilution overnight at 4°C. To characterise the merozoite lysate we obtained from *in vitro* culture, we used sera obtained from rabbits that had been immunised with known recombinant merozoite antigens in previous studies conducted in J. Rayner's laboratories. These antisera included anti-GAP45[326], anti-GAP50[326], anti-MAHRP1, anti-MTIP [326], anti-MyoA [326], anti-MSP3 [327] and anti-EBA175 (Malaria Research and Reference Reagent Resource centre, [www.mr4.org](http://www.mr4.org)). To identify immunogenic merozoite antigens, we used a pool of purified immunoglobulins from malaria immune adults (MIG)[328] and a pool of immunoglobulins from non-malaria exposed Swedish adults (NEG) as the primary antibody. After each incubation step, PVDF membranes were washed thrice with 0.1% Tween20/PBS. Thereafter, anti-rabbit conjugated to horseradish peroxidase (HRP) at a 1:2000 dilution or anti-human IgG conjugated to HRP at various dilutions was incubated for 2 hours at room temperature. Detection of HRP reactivity on PVDF membranes was enabled using the Supersignal West Pico chemiluminescent substrates (Thermoscientific) according to manufacturer's instructions. The two substrates, peroxide and enhancer, were mixed at a 1:1 ratio and incubated with the PVDF membrane for 1 minute. Thereafter, excess substrates were drained and the membrane developed by exposure onto a photographic film (Amersham Hyperfilm ECL) in the dark.



#### **2.4.3. Purified IgG from malaria-immune and non-exposed individuals**

Sera from 17 Swedish adults, who had reported no exposure to malaria nor had travelled to malaria-endemic regions, were selected as our negative controls. These were assayed for reactivity to *P. falciparum* A4 strain schizont extract and apical membrane antigen (AMA1) as a confirmatory test. To test for reactivity, 4HBX immulon plates were coated with 0.5ug/ml of recombinant AMA1 (kindly provided by Dr. Ed Remarque, Biomedical Primate Research Centre, Netherlands) or schizont extract at a 1:8000 dilution overnight at 4°C. Wells were washed four times with 0.05% tween20/PBS before incubation with block buffer (1% non-fat milk/0.05% tween20/PBS) for 5 hours at room temperature followed by incubation with test sera at a 1:1000 dilution overnight at 4°C. The following day, washed wells were incubated with rabbit-antihuman IgG conjugated to HRP for 3 hours at room temperature followed by HRP detection using the sigmaFAST (Sigma Aldrich) detection system. Optical density was measured at 492nm. To purify total immunoglobulin G from the Swedish sera, pooled sera were heat-inactivated at 56°C for 30 minutes and IgG purified using Immunopure Plus Immobilized Protein G Gel (Pierce). Eluted IgG was buffer exchanged into phosphate buffered saline (PBS) thrice, purity confirmed by SDS-page under reducing conditions and stored at -20°C. A reference Malaria Immune Globulin (MIG) reagent (Central laboratory Blood Transfusion Service SRC, Switzerland) that contains 50mg/ml of immunoglobulins (98% IgG) purified from a pool of healthy 834 Malawian adult plasma samples was used as a positive control. This reagent was originally manufactured to test its potential use as an adjunct therapy to quinine in treatment of cerebral malaria[328].

#### **2.4.4. Immuno-electron microscopy on merozoites using MIG and NEG**

To confirm that the merozoite preparation contained merozoites that were differentially reactive with the pool of positive and negative controls, it was prepared for transmission electron microscopy as follows. The *P. falciparum* merozoites were fixed in 4% paraformaldehyde dissolved in 0.1M phosphate buffer

at pH 7.4 for 1 hour at room temperature with gentle rotation. They were then rinsed three times in buffer and infiltrated with 1% and then 10% gelatin before immersion in 2.3M sucrose in phosphate buffer overnight at 4°C for cryoprotection. The frozen samples were prepared by mounting onto aluminium pins and rapidly immersing in liquid nitrogen in preparation for ultrathin 80 nm sectioning on a Leica EM FC6 ultramicrotome. These ultra thin sections were labeled using purified immunoglobulins from non-exposed individuals (NEG) at a 1:500 dilution and MIG at a 1:10000 dilution followed by a goat anti-human IgG (H &L) conjugated to 10nM gold (Abcam). Imaging was performed on an FEI 120kV Spirit Biotwin with a Teitz F4.15 CCD camera.

#### ***2.4.5. Immunoprecipitation of proteins extracted from merozoites***

The immunoprecipitation protocol was tested using parasite culture supernatant obtained following schizont rupture, as this was more readily accessible in a soluble form compared to proteins extracted from purified merozoite lysates. Once the protocol was optimised, it was adapted for use with extracted merozoite proteins.

To optimise the protocol, 300µl of spent culture supernatant was incubated with 200µl of MIG at a 50µg/ml concentration for 1 hour at room temperature with gentle rotation. Fifty microliters of magnetic beads coupled to Protein G (Dynabeads Protein G, Invitrogen) were washed thrice with a citrate-phosphate buffer, pH 5.0 (wash buffer) using a magnet (DynaMag-2, Invitrogen). The antigen-antibody mixture was added to the washed dynabeads and incubated for 2 hours at room temperature with rotation. Eppendorf tubes that contained Protein G coupled to magnetic beads were placed alongside a magnet (DynaMag-2, Invitrogen) for 10 minutes, to allow attachment of beads and the collection of the flow-through fraction. Dynabeads were washed four times with wash buffer by vortexing, before a final 20-minute incubation with 25µl of elution buffer (0.1 M citrate, pH 2-3). Five fractions were subsequently separated on a 4-12% gradient SDS gel and western blots conducted as has been described in section 2.4.2. These fractions

included: 1) the test IgG used in the immunoprecipitation experiments, 2) the culture supernatant containing immunogenic antigens 3) the flow-through following antigen-antibody binding, 4) four washes and 5) the eluates with the immunoprecipitated antigens. Having validated the immunoprecipitation protocol, I adapted it for use with parasite proteins extracted from merozoites with the following changes. Merozoite proteins were extracted using the radio immunoprecipitation assay (RIPA) buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH. 8.0) by gentle agitation at 4°C for 1 hour. The extracted proteins were collected in supernatant, after a centrifugation step at 20000xg for 30 minutes at 4°C. Soluble merozoite proteins in the supernatant were incubated with purified immunoglobulins MIG and NEG at a final concentration of 2500µg/ml and immunoprecipitated as has been described above.

#### ***2.4.6. Bi-dimensional gel electrophoresis coupled to fluorescent western blot***

An alternative method to identifying immunogenic merozoite antigens was tested. This was a two-step procedure in which merozoite proteins were first separated by 2-dimensional gel electrophoresis and subsequently detected in a western blot performed using MIG and NEG (2DE-blot). Two-dimensional gels were made using labeled and unlabeled protein samples obtained from merozoites (3D7 strain and KIL9605 isolate). This involved protein separation in the first dimension by isoelectric focusing on a 13 cm long pH 3-10NL Immobiline Drystrip (GE Healthcare) using the Protean i12 IEF cell system (BioRad) followed by separation in the second dimension by SDS-PAGE gel electrophoresis.

##### ***2.4.6.1. Separation in the first dimension by isoelectric focusing***

Prior to separation in the first dimension, merozoites were first resuspended in extraction buffer (6M Urea, 2M Thiourea, 4% CHAPS, 5mM Magnesium acetate, 10mM Tris pH 8.5.) vortexed and sonicated and extracted proteins quantified using the RC DC protein assay (BioRad). For each 2D-gel, 80µg of protein was Cy5 labelled with 250 picomoles of Chromis 645 dye (Cyanagen, Italy) by incubation

for 30 minutes at room temperature and in the dark followed by a 10-minute incubation with 10mM lysine to quench the reaction. An equal volume of 2X sample buffer containing ampholytes (8M Urea, 4% CHAPS, 2% DTT, 2% IPG buffer 3-10NL GE Healthcare Life Sciences) was added and incubated for 15 minutes in the dark followed by a top up to 250µl with De-streak rehydration solution (GE Healthcare). The 13cm Immobiline DryStrips were rehydrated overnight with the protein solution in the dark after covering the strip with mineral oil to prevent dessication. Thereafter iso-electric focusing was done using a step voltage gradient (50V for 10 hours, 500V for 1 hour, 1000V for 1 hour and 8000V for 48000 volthours) using the Prtean i12 IEF Cell (BioRad).

#### *2.4.6.2. Separation in the second dimension by SDS gel electrophoresis*

The resolved proteins on the 13cm strip were reduced and alkylated prior to separation in the second-dimension by SDS-gel electrophoresis as follows. The resolved proteins on the 13cm strip were reduced for 15 minutes in equilibration reducing buffer (6M Urea, 75mM Tris pH 8.8, 30% glycerol, 2% SDS and 1% DTT). Thereafter the strip was alkylated for 15 minutes in alkylation buffer (6M Urea, 75mM Tris pH 8.8, 30% glycerol, 2% SDS and 2.5% iodoacetamide), and then separated in the second dimension on a 12% SDS-PAGE gel (13cm X 13 cm) using the Hoefer SE 600 vertical unit (GE Healthcare) at 20°C, 20mA for 15 minutes and 40mA until the dye front reached the bottom of the gel. The gel with the unlabeled protein was stained with silver, while those with Cy5 labeled proteins were scanned on a Typhoon 9400 laser scanner (GE healthcare) before transfer onto nitrocellulose membranes for probing with MIG and NEG IgG.

#### *2.4.6.3. Western blot detection of immunogenic antigens separated by 2D-gels*

To identify immunogenic antigens, merozoite proteins resolved in 2D-gels were transferred onto nitrocellulose membranes as has been described in section 2.4.2. The nitrocellulose membrane was blocked for 1 hour at room temperature with 5% non-fat milk/0.1% Tween 20 followed by incubation with MIG or NEG overnight at 4°C at a 1:15000 and 1:5000 dilution respectively. Membranes were washed thrice

with 0.1%PBS/Tween 20 and incubated with goat anti-human IgG-Fc FITC conjugated secondary antibody at 1:500 dilution in the dark. Membranes were washed and scanned on a Typhoon 9400 laser scanner (GE healthcare).

#### *2.4.6.4. Identification and preparation of immunogenic antigens for mass-spectrometry analysis*

Images of the scanned gels, membranes and silver-stained gels were analysed using SpotMap (TotalLab) for alignment and MIG recognised spots were manually excised from the silver-stained gel for mass-spectrometry. Immunogenic spots were excised from 2D-gels, pooled and destained. The proteins were then reduced with 10mM TCEP for 30 minutes at 56°C and alkylated with 55mM iodoacetamide for 45 minutes in the dark. Thereafter, proteins were subjected to enzymatic digestion with trypsin overnight at 37°C at a 1:30 enzyme-protein ratio. The peptides present in the supernatant following trypsin digestion were collected and an equal volume of 100% acetonitrile (ACN) added. The supernatant was vacuum dried and resuspended in 50% acetonitrile/50% of 0.5% formic acid and stored at -20°C. Prior to mass-spectrometry analysis, peptides in supernatant were vacuum dried, resuspended in 65%acetonitrile/35%H<sub>2</sub>O/0.1% formic acid, filtered and flow through vacuum dried and resuspended in 0.5% formic acid.

#### *2.4.7. LC-MS/MS analysis, database search and in-silico analysis of protein sequences*

Peptides obtained from excised 2D spots were resuspended in 40µl of 0.5% formic acid and analysed using an Ultimate 3000 Nano/Capillary LC System (Dionex) coupled to an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher) equipped with a nanospray source. The peptides were first loaded and desalted on a PepMap C18 trap column (0.1mm id x 20mm, 5µm, Dionex) for 20 minutes using a 10µl/min flow rate prior to loading on the separation column (PepMap 75µm id x 25cm column with 5µm particle size, Dionex). The samples were separated over a 120-minute long-linear gradient changing from 100% ACN/0.1%v/v formic acid (FA) in water) to 5-45% of buffer B (80%ACN/0.1%FA) before a final elution

using 95% of buffer B. The Orbitrap mass spectrometer was operated in the “top 15” data-dependant acquisition mode while the preview mode was enabled. The MS full scan was set at m/z of 380-1600 with a resolution at 30,000 and at m/z 400 and the automatic gain control (AGC) set at  $1 \times 10^6$  with a maximum injection time of 200 msec. The 15 most abundant multiply-charged precursor ions, with a minimal signal above 3000 counts, were selected for CID fragmentation (MS/MS) in the LTQ Velos ion trap, which has the AGC set at 5000 and a maximum injection time at 100ms. The raw files were processed in Proteome Discoverer (V1.4) (Thermo Fisher) using Mascot (V2.5) (Matrix Science) as the protein database search engine with the following parameters: Enzymatic digestion was set to trypsin with a maximum of 2 missed cleavages sites; peptide mass tolerance search was set at 20 ppm; MS/MS fragment mass tolerance at 0.50 Da. Variable modifications for Acetyl (Protein N-term), Carbamidomethyl (C), Deamidated (NQ), and Oxidation (M). The protein databases were downloaded from Uniprot (October 2014) plus the common contaminating database. FDR setting was based on PEP at 0.01 in Percolator, and proteins with high confidence peptides were reported.

The proteins identified by the database search were subjected to *in silico* analyses using algorithms for detecting a predicted signal peptide and transmembrane domains <http://www.cbs.dtu.dk/services/SignalP>, <http://www.cbs.dtu.dk/services/TMHMM/> respectively. The transcript levels of identified genes were derived from microarray transcriptome data available on PlasmoDB (<http://plasmodb.org/plasmo/>). Gene ontology cellular component enrichment analysis was performed using PlasmoDB where the frequency of occurrence of a particular cellular component term within the list of identified proteins is compared to the background frequency of occurrence in the entire organisms set of genes.

#### **2.4.8. Statistical analysis**

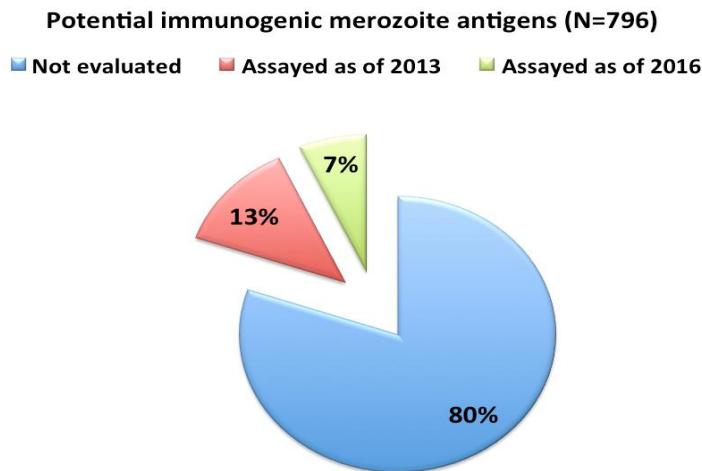
Data analysis was performed using STATA 13 (StatCorp, TX, USA) and GraphPad Prism6 (GraphPad Software, San Diego, California, USA). The Mann-Whitney test was used to compare antibody levels to merozoite antigens in a pool of hyper-immune sera (PHIS) and non-exposed sera. The unpaired t-test was used to compare the mean dN/dS SNP ratio's between parasite proteins identified in the *Pf*3D7 2DE-blots, KIL9605 2DE-blots or in both data sets.

## 2.5. Results

### 2.5.1. Identifying potential immunogenic merozoite antigens using PlasmoDB

I mined data from PlasmoDB to identify antigens that could be accessible to antibodies in circulation, either by being secreted or located on the surface of *P. falciparum* merozoites. I limited the list to parasite proteins whose microarray expression profiles indicated that maximum mRNA transcription occurs in the late-trophozoite, early-late schizogony or merozoite stage of the erythrocytic life cycle [259, 260]. I used the stages of the erythrocytic life cycle as was defined and deposited on PlasmoDB by Le Roch *et al* [260]. In addition, I limited the list of genes to those with a predicted signal peptide and/or predicted transmembrane domains that would indicate the protein could potentially be: i) secreted ii) peripherally associated to or iii) an integral protein found on the merozoite surface. I identified a total of 796 proteins, 550 (69.1%) and 571 (71.7%) of which had a predicted N-terminal signal peptide and at least one predicted transmembrane domain respectively. The median predicted isoelectric point of the list of potential immunogenic merozoite antigens was 8.68 (range: 3.58-11.32) and the median predicted molecular weight was 55 KDa (range: 6-1206). As shown in Figure 2.1, of this list of potential immunogenic merozoite antigens, I identified 157 (19.7%) proteins that had been evaluated for immunogenicity (as of September 2016) using data that is publicly available from peer-reviewed publications [146, 147, 267, 271]. This preliminary analysis indicated that there were potentially many more immunogenic (over 600) merozoite antigens yet to be identified and evaluated.

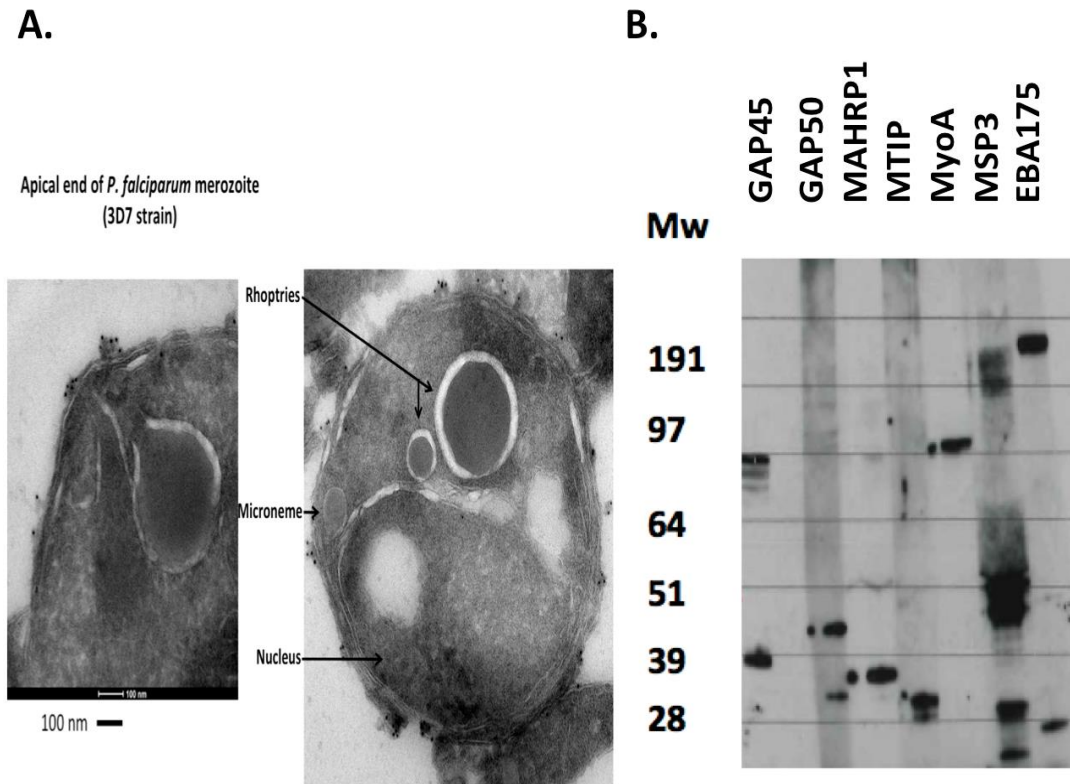




**Figure 2.1: Proportion of merozoite antigens assayed for immunogenicity.** Pie chart showing the proportion of potentially immunogenic merozoite antigens assayed for immunogenicity as of 2013 and 2016.

### ***2.5.2. Visualization of the merozoites isolated from *in vitro* culture***

*P. falciparum* merozoites (3D7 strain) were obtained from *in vitro* culture and imaged under an electron microscope. This was done to confirm that the purification steps resulted in obtaining merozoites. In addition, I tested for reactivity with known merozoite antigen antisera that were readily available. As shown in Figure 2.2A, the characteristic shape of a circular merozoite with a protruding apical end was evident. Similarly, known merozoite organelles were clearly visible including the micronemes located near the apical end, the large rhoptry and rhoptry neck toward the apical end of the merozoite. A large nucleus was also evident below the rhoptries. A panel of rabbit anti-sera that had previously been generated against specific merozoite antigens was used to validate the content of the merozoite pellet material obtained from *in vitro* culture.



**Figure 2.2: Characterising purified *P. falciparum* merozoites.** Characterisation of the purified *P. falciparum* merozoites obtained from *in vitro* culture by transmission electron microscopy and western blot A) Transmission electron microscopy of the apical end of a merozoite and the whole merozoite B) Western blot detection of known merozoite antigens on purified merozoite preparations separated on a SDS-PAGE, using anti-GAP45, anti-GAP50, anti-MAHRP1, anti-MTIP, anti-MyoA, anti-MSP3 and anti-EBA175 antisera.

As shown in Figure 2.2B, all the available antigen-specific sera detected their respective antigens in the merozoite pellet using Western blots. These included anti-GAP50, anti-MTIP, anti-MyoA, anti-MSP3 and anti-EBA175 at the expected molecular weights [326]. Anti-GAP45 showed reactivity at approximately 39kDa and between 64-97kDa while the predicted molecular weight was 45kDa. Reactivity with anti-GAP50, anti-MAHRP1, anti-MTIP, anti-MyoA, anti-MSP3 and anti-EBA175 sera was observed between 39-51, 28-39, 28, 64-97, 39-51 and 97-191 kDa respectively, consistent with their predicted molecular weights. Anti-MAHRP1 sera recognised a protein at the expected predicted molecular weight for MAHRP-1 (29kDa). This protein has been shown to localise to the Maurer's clefts, a parasite-derived vesicle structure in the infected erythrocyte cytosol, and is thought to play a

role in organising and sorting parasite proteins [329]. The presence of MAHRP-1 in the preparation suggests that the purified lysate contain some parasite material from schizonts as part of the debris obtained during filtration to collect merozoites.

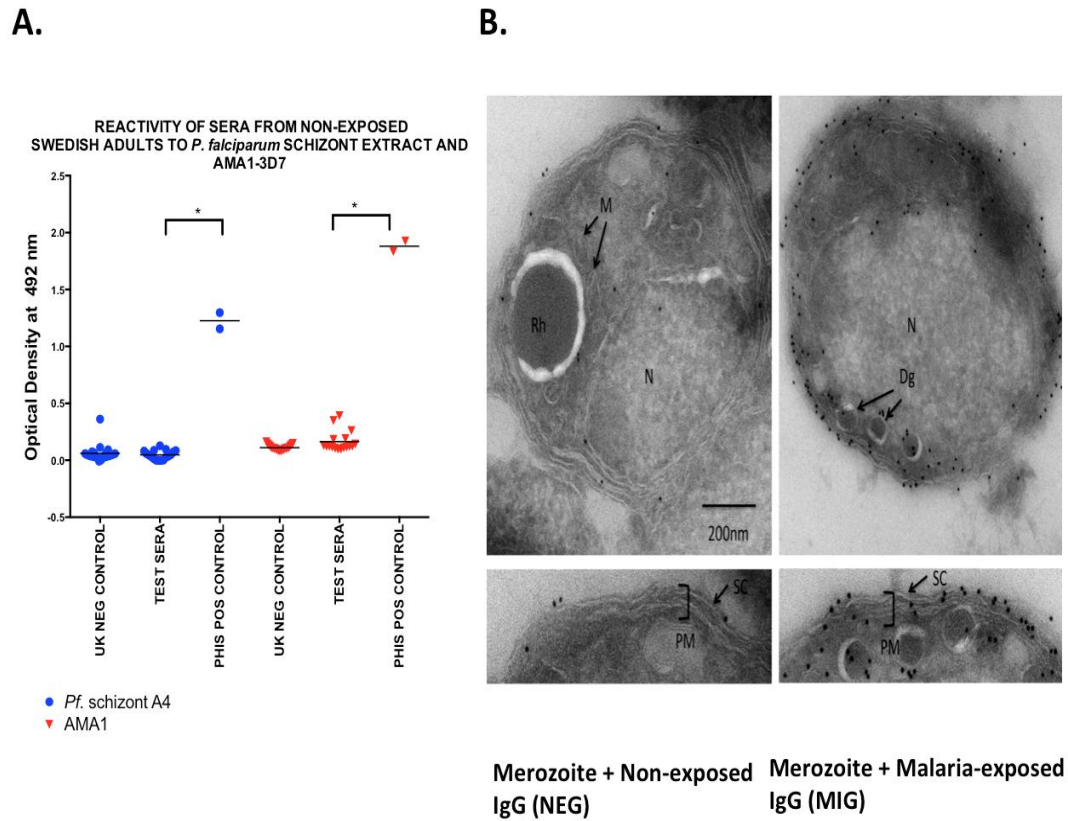
### ***2.5.3. Validating the immunoglobulins from exposed and non-exposed individuals***

To ensure that the positive control immunoglobulins (MIG) recognised merozoite proteins and the negative controls were non-reactive to malaria antigens, I conducted the following tests: i) tested each individual negative control serum samples for reactivity with the immunodominant merozoite antigen AMA1 and schizont extract by ELISA and ii) tested whether purified merozoites would be recognised by the negative and positive control when using immunoelectron microscopy.

As shown in Figure 2.3A, the presence of antibodies to AMA1 and schizont extract, was measured in the negative controls (the test sera), a second pool of non-exposed sera from adults resident in the United Kingdom (UK) and a pool of hyper-immune sera collected from adults living in the malaria-endemic region Kilifi in Kenya. I observed no differences in the levels of reactivity to both schizont extract and AMA1 in the two sets of negative controls. The pool of hyper-immune sera (PHIS) had significantly higher antibody levels to schizont extract compared to Swedish negative control sera (Mann-Whitney test: 1.226 versus 0.046; p-value=0.011). Similarly, reactivity to AMA1 was significantly higher in PHIS compared to Swedish sera (Mann-Whitney test: 1.880 versus 0.123; p-value=0.011).

I then confirmed that purified merozoites were differentially recognised by the positive and negative control. Transmission electron microscopy was performed on purified merozoites incubated with purified immunoglobulins from negative and positive controls (Figure 2.3B). I observed higher reactivity in merozoites incubated with MIG compared to those incubated with negative pool of IgG as shown by dark spots on the surface of the merozoite. This reactivity was localised primarily on the

surface of the merozoites. A low level of reactivity was observed with the negative control IgG that was similarly primarily localised on the surface of the merozoites.

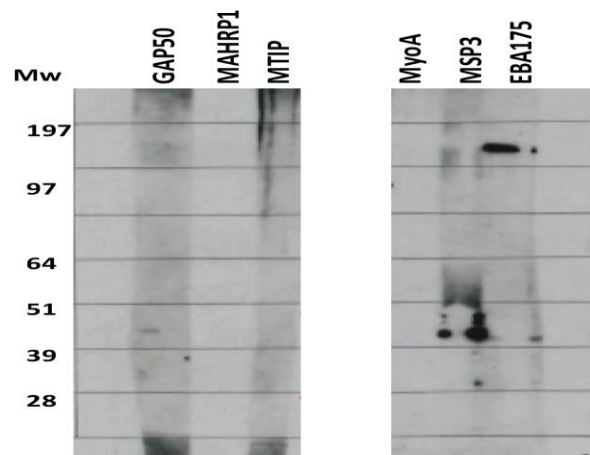


**Figure 2.3: Validating the pool of non-exposed and malaria-exposed purified immunoglobulins.** A) Reactivity of individual Swedish adult samples (TEST) with *Pf* schizont extract and AMA1. A second pool of negative controls and a pool of hyper-immune sera from adults living in Kilifi (PHIS) were included for comparison. B) Transmission electron microscopy image showing reactivity of purified *Pf* merozoites with purified immunoglobulins from non-malaria exposed adults (NEG) and malaria-exposed adults (MIG). M=micronemes. Rh=Rhoptries. N=Nucleus. Dg=Dense granules. PM=Plasma membrane. SC=Surface coat.

#### 2.5.4. Immunoprecipitation of MSP3 using parasite culture supernatant

To optimise the immunoprecipitation protocol using magnetic beads coupled to protein G, I used spent culture supernatant into which parasite proteins were released following merozoite egress from schizonts. Prior to the immunoprecipitation experiment, I tested for the presence of known immunogenic merozoite antigens, released into culture supernatant following schizont rupture

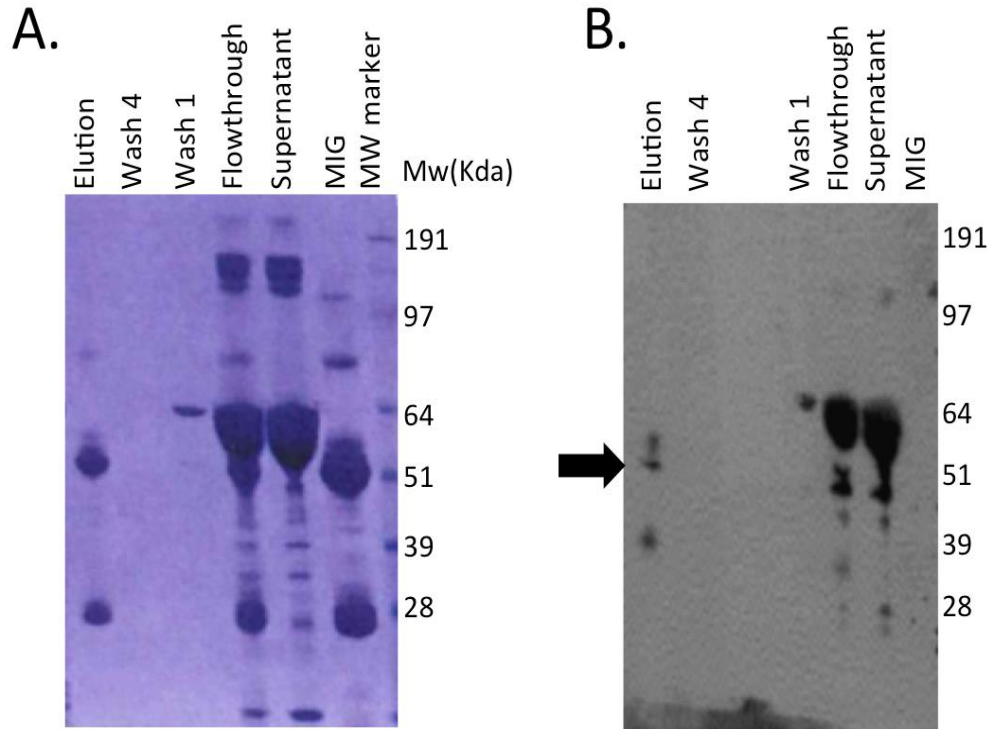
(Figure 2.4). I detected MSP3 (Figure 2.4, lane 5) and EBA175 (Figure 2.4, lane 6) in spent culture supernatant using rabbit anti-MSP3 and anti-EBA175 sera. As expected, no reactivity was observed with anti-GAP50, anti-MAHRP1, anti-MTIP and anti-MyoA as shown in Figure 2.4 lanes 1, 2 and 3 respectively. Three of these antigens (GAP50, MTIP and MyoA) are glideosome proteins located on the inner membrane complex detectable in late schizonts and merozoites that form a complex involved in erythrocyte invasion [326], while MAHRP-1 is a protein localised to the Maurer's Cleft within the infected erythrocytes. As a result of these locations, none of these antigens were expected to be secreted into the parasite culture supernatant following merozoite egress. Having confirmed the presence of the known immunogen MSP3 in the spent parasite culture supernatant, I tested the ability of the pool of purified immunoglobulins (MIG) to immunoprecipitate it alongside other immunogenic merozoite antigens.



**Figure 2.4: Detection of merozoite antigens in culture supernatant.** Western blot detection of known merozoite antigens using spent culture supernatant separated on SDS-PAGE, using lane 1) anti-GAP50 2) anti-MAHRP1 3) anti-MTIP 4) anti-MyoA 5) anti-MSP3 and 6) anti-EBA175 antisera.

To test the ability of MIG to immunoprecipitate MSP3 (a known immunogen), I conducted pull-down experiments using parasite culture supernatant obtained following merozoite egress as the source of MSP3 and other immunogenic antigens. I monitored the experiment by SDS-PAGE (Figure 2.5A) and Western blots (Figure 2.5B). As shown in Figure 2.5B, the MSP3 band at a size between 39-51 KDa was observed in the supernatant fraction, flowthrough as well as in the elution fraction

(as indicated by the arrow). No bands were detected in the fraction containing MIG antibodies or in the washes, implying immunoprecipitation of known immunogenic antigen MSP3 by the pool of immunoglobulins from immune adults.



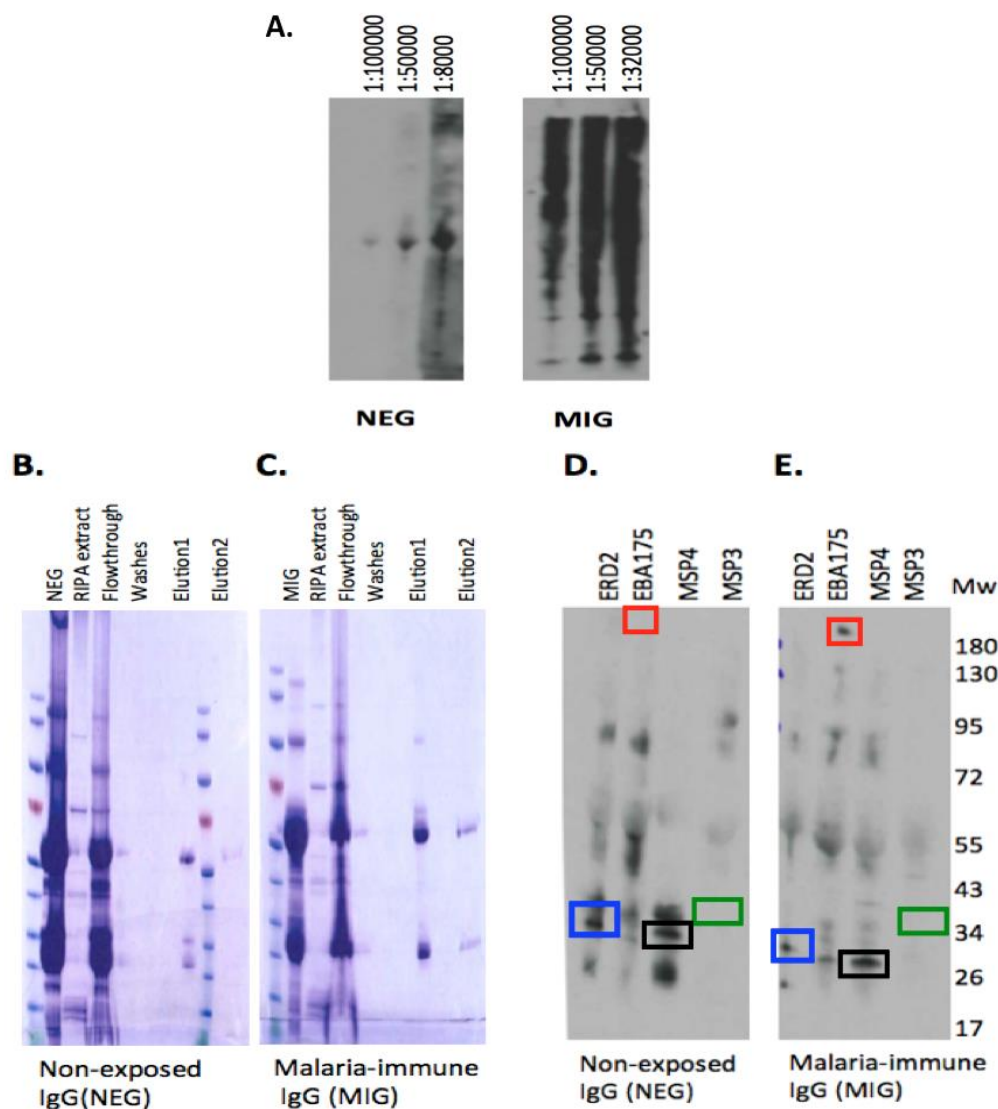
**Figure 2.5: Detection of immunoprecipitated MSP3 from culture supernatant.** Validation of immunoprecipitation protocol using spent culture supernatant. A) Coomassie stained SDS-PAGE showing the proteins present in all fractions collected from immunoprecipitation experiments. B) Western blot detection of MSP3 in the supernatant, flowthrough and elution fractions of the immunoprecipitation experiment shown using the arrow. The Coomassie stained bands at the molecular weights regions corresponding to 50 and 25 KDa are the heavy and light chains of human IgG while the other visible bands correspond to parasite proteins.

I then conducted immuno-precipitation experiments using RIPA extracted merozoite protein using MIG and non-exposed IgG as a control. Prior to the immunoprecipitation, I confirmed that non-exposed IgG and MIG differentially recognised the merozoite proteins on a Western blot as shown in Figure 2.6A. Merozoite proteins were detected by MIG even at a dilution of 1:100,000 while no reactivity was evident with NEG at a dilution of 1:8000. Thereafter, I immunoprecipitated RIPA extracted merozoite proteins using NEG and MIG and

probed the elutions for the presence of known immunogenic antigens and the absence of non-immunogenic proteins by western blot. Figure 2.6B and C show Coomassie stained SDS-gels of fractions collected from immunoprecipitation experiments using NEG and MIG respectively. These gels confirmed that the RIPA extraction protocol was successful in extracting merozoite proteins of varying sizes. Similarly, proteins were evident in the eluates from NEG (Figure 2.6B) and MIG (Figure 2.6C) immunoprecipitation experiments including the heavy and light chains of the respective IgG's at 50 and 25 KDa. The binding of antibodies to Protein G is disrupted alongside immunoprecipitated proteins during the elution step, so elution of IgG bound to the column is expected.

I tested the eluates for immunoprecipitation of known immunogenic merozoite antigens namely MSP3, MSP4 and EBA175. I expected that these antigens would be detectable in the eluate fraction from MIG and absent in the NEG immunoprecipitation experiment. I also tested for the presence of ERD2, a non-immunogenic internal merozoite antigen that would serve as a negative control and expected to be absent in both eluates. Surprisingly, as shown in Figure 2.6D and E, the known immunogenic merozoite antigen MSP4 was detected in elutions from both NEG and MIG immunoprecipitation experiments (Figure 2.6D and E, shown in the black squares). A third immunogenic antigen MSP3 was not detected in either of the eluates (Figure 2.6D and E, shown in the green squares). Lastly, the non-immunogenic antigen ERD2 was detected in both elutions (Figure 2.6D and E, shown in the blue squares). EBA175 was present in the eluate from MIG immunoprecipitation and absent in the NEG experiment as was expected (Figure 2.6D and E, shown in the red squares). These results indicated that there was some level of non-specific immunoprecipitation evidenced by ERD2 and MSP4. MSP3 was not detected in eluates from both MIG and NEG immunoprecipitation experiments.



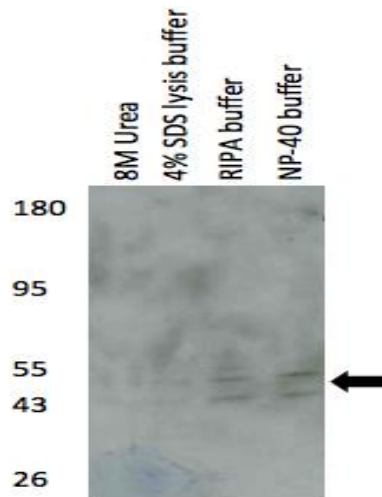


**Figure 2.6: Immunoprecipitation of merozoite antigens.** Immunoprecipitation of RIPA extracted merozoite proteins using non-exposed IgG (NEG) and malaria-immune IgG (MIG). A) Recognition of merozoite proteins by NEG at a dilution of 1:8000, 1:50000 and 1:100000. Recognition of merozoite proteins by MIG at a dilution of 1:32000, 1:50000 and 1:100000. B) Coomassie stained SDS gel of fractions obtained from immunoprecipitation of RIPA extract using NEG. C) Coomassie stained SDS gel of fractions obtained from immunoprecipitation of RIPA extract using MIG. Western blot detection of MSP3 (green squares), MSP4 (black squares), EBA175 (red squares) and ERD2 (blue squares) in elution's following immunoprecipitation with NEG (D) and MIG (E).

One possible explanation for not detecting MSP3 in either eluate would be that RIPA buffer did not extract and solubilise MSP3. However as shown in Figure 2.7, MSP3 was detectable using rabbit anti-MSP3 in merozoite proteins extracted with



RIPA and NP-40 buffer but not detected in extracts using 4% SDS-lysis or the 8M Urea buffer.



**Figure 2.7: Detection of MSP3 solubilised using different extraction buffers.** Detection of MSP3 using rabbit anti-MSP3 sera in merozoite proteins extracted with 8M Urea, 4% SDS lysis buffer, RIPA buffer and NP-40 buffer.

The problems with background in the immunoprecipitation experiments, as well as the suggestion that the immunoprecipitation is not highly efficient, as evidenced by the absence of precipitation of MSP3, prompted me to explore bi-dimensional gel electrophoresis coupled to Western blotting as an alternative strategy.

#### **2.5.5. Identification of immunogenic merozoite antigens using 2DE-Western Blots: 3D7 strain**

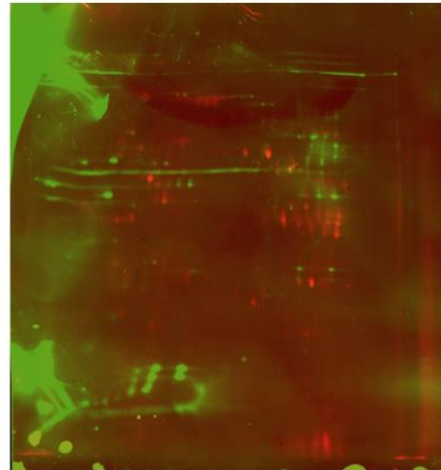
*P. falciparum* merozoite antigens from the 3D7 strain were analysed by bi-dimensional isoelectric focusing gel electrophoresis using a pH gradient ranging from pH 3-10 and silver-stained after the second dimension separation by SDS as shown in Figure 2.8A. Merozoite proteins that were recognised by MIG or NEG were detected using FITC conjugated anti-human antibody visible as green spots and shown in Figure 2.8B and C respectively. As described in detail in section 2.4.6.1, proteins extracted from merozoites were Cy5 labelled (red) prior to resolution by 2DE. These proteins were then probed for reactivity with MIG or

NEG and bound IgG were detected using a secondary antibody that was FITC labelled (green). As a result, scanning of the 2DE-Western blots revealed two colours, red spots indicated resolved merozoite proteins that did not react with IgG while green spots indicated proteins bound by IgG. The degree of Cy5 labelling is likely to reflect the quantity of lysine residues present in each protein as the dyes covalently attach to the free amine group on the N-terminal or lysine residues on proteins.

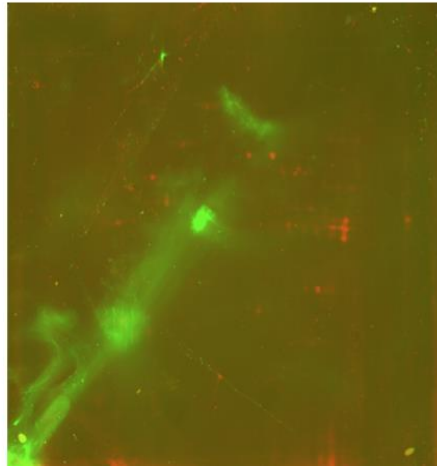
**A. Silver-stained 2D-gel**



**B. Western Blot using MIG**



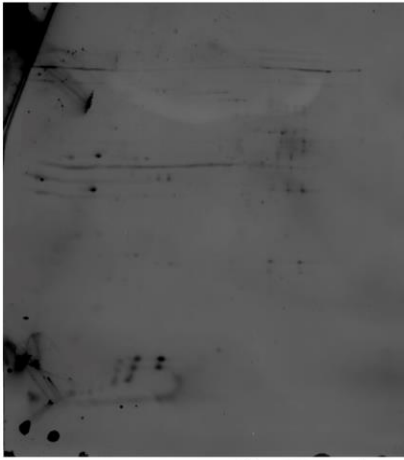
**C. Western Blot using NEG**



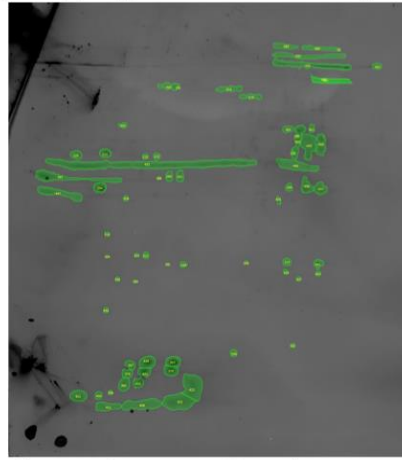
**Figure 2.8: Detection of immunogenic 3D7 antigens by 2DE.** Bi-dimensional gel electrophoresis of *P.falciparum* 3D7 strain merozoite antigens and reactivity with MIG and NEG. A) Silver-stained image of merozoite antigens separated by two-dimensional gel electrophoresis (2D-gels). B). Fluorescent western blot image of MIG reactive merozoite antigens C) Fluorescent western blot detection of NEG reactive merozoite antigens. Antibody reactive proteins are shown as green spots while red spots are Cy5 labelled merozoite proteins that are not bound by antibodies.

Distinct green fluorescent spots were visible in western blot with MIG (Figure 2.8B and Figure 2.9A) and these were mapped using the SpotMap image analysis software onto the silver-stained gel (Figure 2.9B and C). A total of 69 spots or regions were mapped and manually excised from the silver-stained gel for identification by mass-spectrometry.

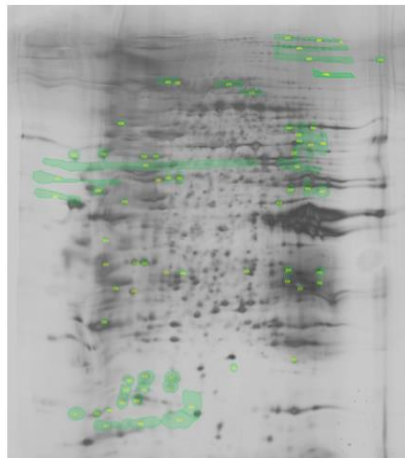
**A. Western Blot using MIG**



**B. Mapping MIG reactive antigens on Blot**



**C. Mapping MIG reactive antigens on 2D-gel**

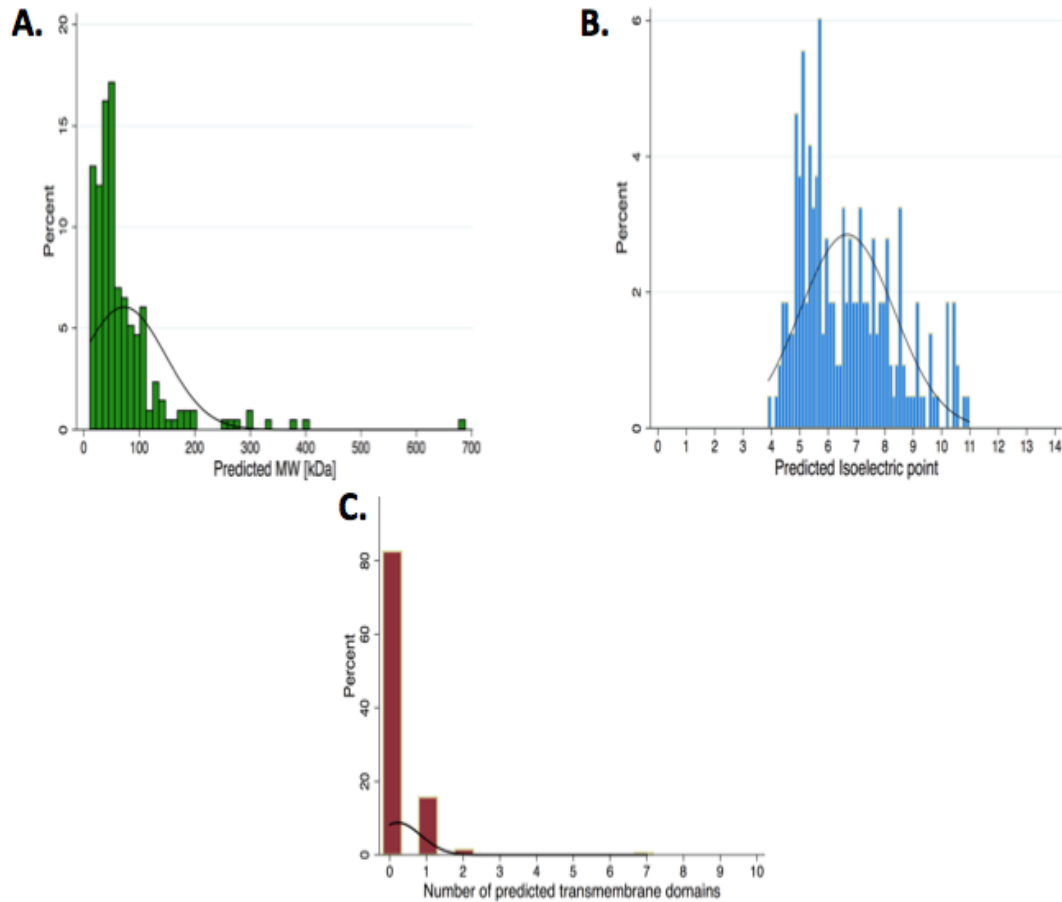


**Figure 2.9: Mapping immunogenic antigens on a 2DE gel.** Mapping of immunogenic merozoite antigens onto silver-stained gel for manual excision and identification by mass-spectrometry. A) Western blot image of antigens recognised by MIG B) Mapping of spots recognised by MIG using SpotMap, image analysis tool C) Mapping of spots recognised by MIG onto the silver-stained gel image using SpotMap, image analysis tool. Antibody reactive proteins are shown as green spots.

#### ***2.5.6. Protein characteristics (3D7 strain): signal peptides and transmembrane domains***

The proteins corresponding to regions of the Western blot recognised by MIG were excised from silver-stained gels and identified by mass-spectrometry. These yielded a list of 216 unique UniProt identifiers, which mapped onto 210 unique proteins in the PlasmoDB database (Appendix 2.1). I analysed the list of proteins obtained for their predicted isoelectric points and molecular weights, as I had used a pH gradient of limited to pH: 3-10 for separation in the first dimension and a 12% SDS-gel for the second dimension limited to separation of proteins between 10-250 KDa.

As shown in Figure 2.10A and B, the identified proteins had a predicted median molecular weight of 51.0 KDa (range: 11.4-688.9) and a predicted median isoelectric point of 6.38 (range: 3.89-10.98). Searching the SignalP and TMHMM webservers revealed that forty-nine (20.5%) and 37 (17.6%) of the identified proteins were predicted to have a signal peptide and transmembrane domain(s), respectively. A known limitation of 2DE is the inability of the extraction buffers to solubilise membrane proteins. To evaluate whether membrane proteins were underrepresented in my findings, I compared the occurrence of features within my list and the total 3D7 predicted genes. The proportion of proteins with transmembrane domains identified by 2D-blot was statistically lower than their occurrence within protein coding genes in the 3D7 genome (genome versus 2D-blot: 30.6% versus 17.6%; p-value=0.0001). No differences were observed with proteins predicted to have a signal peptide (genome versus 2D-blot: 19.3% versus 20.5%; p-value=0.6712). Of those proteins with predicted transmembrane domains, 33 (15.7%), 3 (1.43%) and 1 (0.48%) were predicted to have one, two and seven transmembrane domains as shown in Figure 2.10C.



**Figure 2.10: Characteristic of proteins identified by mass-spectrometry spots picked from the 2D-Western Blots.** Histograms showing the distribution of the predicted molecular weights (A), isoelectric points (B) and number of transmembrane domains (C) in the proteins identified by mass-spectrometry.

### ***2.5.7. Protein characteristics (3D7 strain): predicted cellular localization and transcriptional profiles***

To further analyse the characteristics of the proteins identified, I obtained data from PlasmoDB with information on the transcriptional profiles during the erythrocytic cycle and the predicted gene ontology for each of the antigens. The transcriptome data collected indicated the maximal transcriptional timing across the 48-hour asexual cycle. The gene ontology analysis on PlasmoDB provides terms associated with each gene's products that represent its predicted properties such as cellular location or the biological processes associated with the gene product (<http://geneontology.org>). A total of 24 predicted cellular GO terms were obtained

which were classified into four major subcellular localizations namely: nucleus-, cytoplasm-, mitochondrial- and membrane- associated localization as shown in Table 2.1.

**Table 2.1: Table showing the classification of the predicted cellular localization gene-ontology terms for proteins identified in the 2DE-Western Blots experiments: 3D7 strain**

<b>The classification of the 24 cellular GO terms into 4 broad cellular localization terms</b>	
<b>Nucleus-associated</b>	PCNA complex
	cytoplasm, nucleus
	endoplasmic reticulum
	eukaryotic translation elongation factor
	intracellular, ribosome
	nucleus
	nucleosome, nucleus
	ribosome
	ribosome, small ribosomal subunit
	small ribosomal subunit
	eukaryotic translation elongation factor 1 complex
<b>Cytoplasm-associated</b>	cAMP-dependent protein kinase complex
	cytoplasm
	intracellular
	intracellular, membrane
	oxoglutarate dehydrogenase complex
	phosphopyruvate hydratase complex
	prefoldin complex
	proteasome core complex
	protein complex
<b>Mitochondrial-associated</b>	mitochondrial envelope
	mitochondrial outer membrane
<b>Membrane associated</b>	membrane
	myosin complex
<b>No predicted GO term</b>	null

As shown in Table 2.2, the majority of proteins identified 164 (75.9%) had no predicted localisation. Twenty (9.3%) and 19 (8.8%) proteins, were predicted to be localised to the nucleus and the cytoplasm, respectively. Two proteins (0.9%) were considered mitochondrial-associated and five (2.3%) as membrane associated proteins. The median maximal transcription hours post invasion (hpi) was 29 hours.

Proteins were classified as having their maximal transcription between 1-16 hpi, 17-34 hpi and 35-48 hpi representing the ring, trophozoite and schizont stages of the erythrocytic life cycle. The majority (78.1%) of identified proteins showing elevated transcription levels in the 17-34 hpi and 35-48 hpi as shown in Table 2.2.

**Table 2.2: Table showing predicted gene-ontology terms and maximal transcription profiles for proteins identified in the 2DE-Western Blot experiments: 3D7 strain**

<b>Number of proteins categorised into broad predicted cellular localization</b>	
<b>Predicted cellular location</b>	<b>N (%)</b>
Nucleus associated	20 (9.3)
Cytoplasm	19 (8.8)
Mitochondria	2 (0.9)
Membrane, extracellular	5 (2.3)
Null	164 (75.9)
Data not available	6 (2.8)
<b><i>P. falciparum</i> maximal expression timing over the 48 hour erythrocytic cycle</b>	
<b>Hours post merozoite invasion (hpi)</b>	<b>N (%)</b>
1-16	40 (19.1)
17-34	83 (39.5)
35-48	81 (38.6)
Data not available	6 (2.9)

#### ***2.5.8. Comparison of identified antigens in the 2DE-Western Blot (3D7 strain) and known immunogenic antigens***

Of the 210 proteins identified in the spots excised from 2DE gels, I found that 35 (16.7%) of the targets had been previously shown to be immunogenic in peer-reviewed publications [146, 147, 265, 267, 271, 292]. These include known immunogenic merozoite antigens such as Merozoite Surface Protein 1, 2, 3, 6, 7 and 9, Apical Membrane Antigen 1 (AMA1), GPI-anchored Micronemal Antigen (GAMA), Glutamate Rich Protein (GLURP), High Molecular Weight Rhoptry Protein 3 (RhopH3) and Serine Repeat Antigen 4 and 5 (SERA 4 and 5). The full list of antigens identified in the 3D7 2DE-Western Blots and previously shown to be immunogenic is provided in Table 2.3. The proportion of the entire protein that was

identified by mass-spectrometry, as well as the number of unique peptides used to identify the protein, are shown in the columns labeled sequence coverage and unique peptides respectively. The majority of the antigens that I identified, 175 (83.3%), do not appear to have been studied in the context of naturally acquired immunity to malaria.

**Table 2.3: Table showing proteins identified in the 2DE-Western Blot and previously shown to be immunogenic: 3D7 strain**

PlasmoDB identifier	Product description	Sequence Coverage	Unique peptides	Predicted signal peptide	Predicted transmembrane domains
PF3D7_0818200	14-3-3 protein (14-3-3I)	66.41	16	No	No
PF3D7_1232100	60 kDa chaperonin (CPN60)	15.74	8	Yes	Yes
PF3D7_0828800	GPI-anchored micronemal antigen (GAMA)	5.96	3	Yes	Yes
PF3D7_1311800	M1-family alanyl aminopeptidase (M1AAP)	0.92	1	No	Yes
PF3D7_0401800	Plasmodium exported protein (PHISTb)(PfD80)	3.21	1	No	Yes
PF3D7_0801000	Plasmodium exported protein (PHISTc), unknown function	0.74	1	No	Yes
PF3D7_1149000	Antigen 332, DBL-like protein (Pf332)	0.34	2	No	No
PF3D7_1133400	Apical membrane antigen 1 (AMA1)	1.93	1	Yes	Yes
PF3D7_1014100	Conserved Plasmodium protein, unknown function	0.39	1	Yes	No
PF3D7_1468100	Conserved Plasmodium protein, unknown function	1.72	2	No	No
PF3D7_1320800	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	3.56	1	No	No
PF3D7_1401400	Early transcribed membrane protein 14.1 (ETRAMP14)	12.15	1	Yes	Yes
PF3D7_0532100	Early transcribed membrane protein 5 (ETRAMP5)	28.18	6	Yes	Yes
PF3D7_1222300	Endoplasmic, putative (GRP94)	21.07	13	Yes	No
PF3D7_1035300	Glutamate-rich protein (GLURP)	0.89	1	Yes	No
PF3D7_0818900	Heat shock protein 70 (HSP70)	71.49	71	No	No
PF3D7_0905400	High molecular weight rhoptry protein 3 (RhopH3)	9.7	7	Yes	No



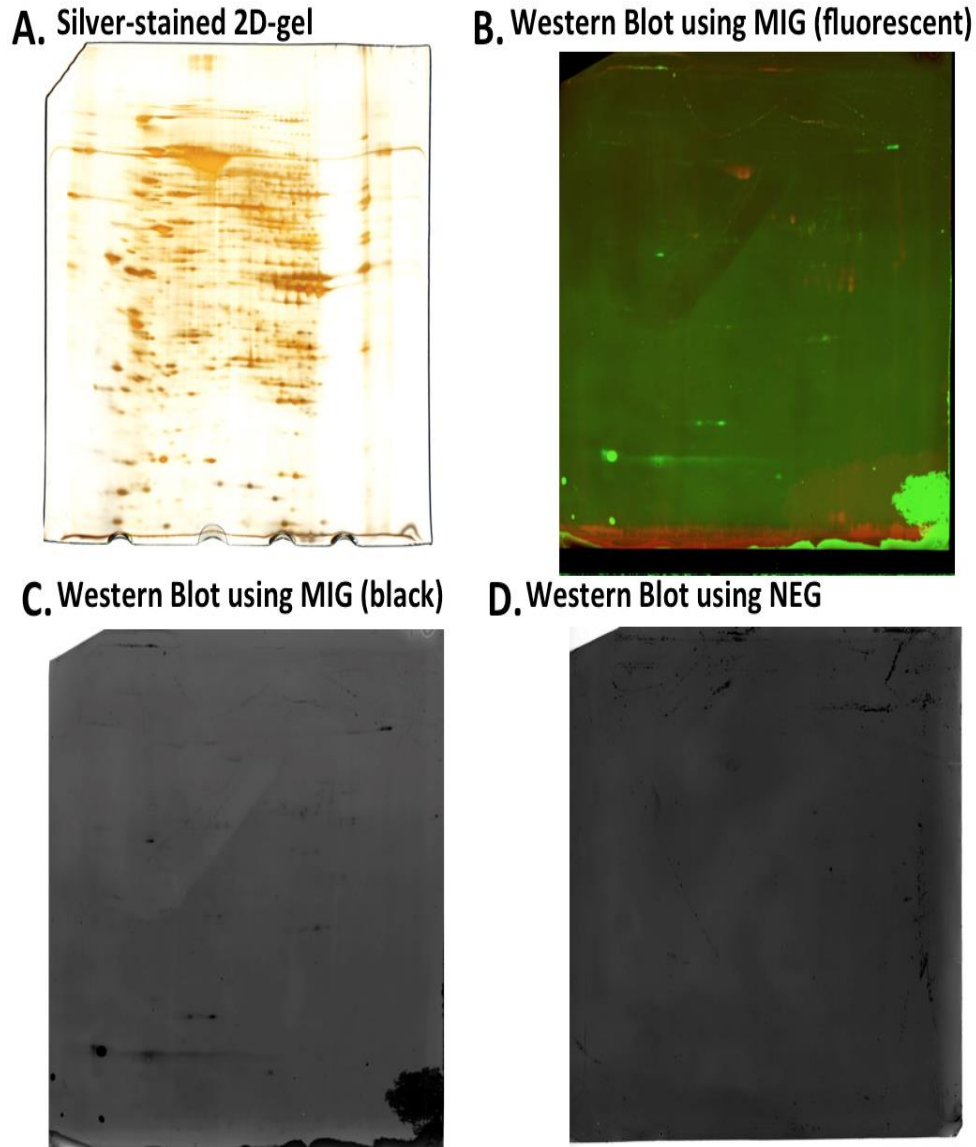
PlasmoDB identifier	Product description	Sequence Coverage	Unique peptides	Predicted signal peptide	Predicted transmembrane domains
PF3D7_0532400	Lysine-rich membrane-associated PHISTb protein (LyMP)	2.27	1	No	Yes
PF3D7_0500800	Mature parasite-infected erythrocyte surface antigen, erythrocy 2 (MESA)	8.86	9	No	No
PF3D7_0930300	Merozoite surface protein 1 (MSP1)	41.98	76	Yes	Yes
PF3D7_0206800	Merozoite surface protein 2 (MSP2)	22.06	4	No	No
PF3D7_1035400	Merozoite surface protein 3 (MSP3)	44.92	18	Yes	No
PF3D7_1035500	Merozoite surface protein 6 (MSP6)	32.35	13	No	No
PF3D7_1335100	Merozoite surface protein 7 (MSP7)	54.7	26	Yes	No
PF3D7_1228600	Merozoite surface protein 9 (MSP9)	1.62	1	Yes	No
PF3D7_1129100	Parasitophorous vacuolar protein 1 (PV1)	46.68	16	Yes	No
PF3D7_1467900	Rab GTPase activator, putative	0.66	1	No	No
PF3D7_0935900	Ring-exported protein 1 (REX1)	15.01	3	No	Yes
PF3D7_1149200	Ring-infected erythrocyte surface antigen	1.1	1	No	No
PF3D7_0207700	Serine repeat antigen 4 (SERA4)	9.46	6	Yes	No
PF3D7_0207600	Serine repeat antigen 5 (SERA5)	19.66	16	Yes	No
PF3D7_1414400	Serine/threonine protein phosphatase PP1 (PP1)	3.95	1	No	No
PF3D7_0702400	Small exported membrane protein 1 (SEMP1)	17.89	2	No	Yes
PF3D7_1436300	Translocon component PTEX150 (PTEX150)	8.36	8	Yes	No
PF3D7_1008700	Tubulin beta chain	14.16	6	No	No

### **2.5.9. Identification of immunogenic merozoite antigens using 2DE-Western Blots: KIL9605 isolate**

As long-term lab adapted isolates such as the 3D7 strain are known to undergo spontaneous gene deletions during *in vitro* culture [330, 331], and have not been exposed to immune pressure for a considerable period, I hypothesised that a recently lab-adapted isolate (KIL9605; obtained from a cerebral malaria patient admitted to Kilifi County Hospital, Kenya) might express a more diverse array of merozoite antigens.

Purified *P. falciparum* merozoite antigens from KIL9605 were analysed by bi-dimensional isoelectric focusing gel electrophoresis using a pH gradient ranging from pH 3-10 and silver-stained after the second dimension separation by SDS as shown in Figure 2.10A. and as described above for the 3D7 strain. Spots or regions recognised by MIG are shown in Figure 2.11B and C while the negative control IgG was not reactive as shown in Figure 2.11D.

I mapped immunoreactive spots using the SpotMap image analysis software onto the silver-stained gel (Figure 2.12A, B and C). A total of 117 spots or regions were mapped and manually excised from the silver-stained gel and identified by mass-spectrometry. 271 unique uniprot identifiers were identified that mapped to 262 PlasmoDB unique identifiers and available in Appendix 2.2. As hypothesised, this was higher than the number of targets identified in the 3D7 immunoblot experiment.

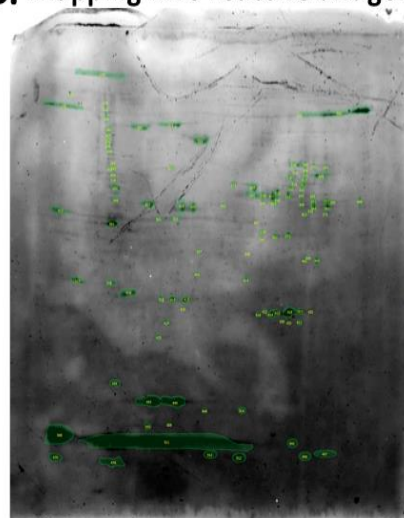


**Figure 2.11: Detection of immunogenic KIL9605 antigens by 2DE.** Bi-dimensional gel electrophoresis of *P.falciparum* KIL9605 strain merozoite antigens and reactivity with MIG and NEG. A) Silver-stained image of merozoite antigens separated by two-dimensional gel electrophoresis (2D-gels). B). Fluorescent western blot image of MIG reactive merozoite antigens C) Western blot image of MIG reactive merozoite antigens D) Western blot image of NEG reactive merozoite antigens. Antibody reactive proteins are shown as green spots while red spots are Cy5 labelled merozoite proteins that are not bound by antibodies.

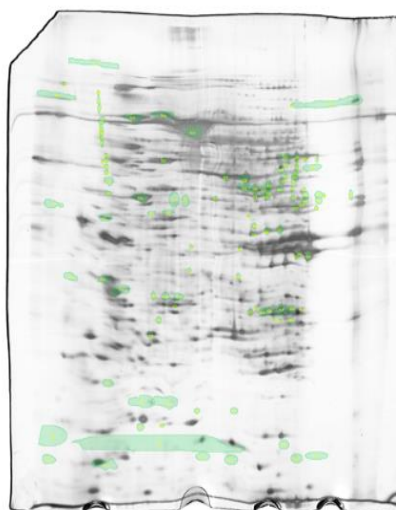
**A. Western Blot using MIG**



**B. Mapping MIG reactive antigens on Blot**



**C. Mapping MIG reactive antigens on 2D-gel**



**Figure 2.12: Mapping immunogenic antigens on a 2DE gel.** Mapping of immunogenic merozoite (KIL9605 strain) antigens onto silver-stained gel for manual excision and identification by mass-spectrometry. A) Western blot image of antigens recognised by MIG B) Mapping of spots recognised by MIG using SpotMap, image analysis tool C) Mapping of spots recognised by MIG onto the silver-stained gel image using SpotMap, image analysis tool. Antibody reactive proteins are shown as green spots.

#### ***2.5.10. Protein characteristics (KIL9605 isolate): signal peptides, transmembrane domains and transcriptional profiles***

Similar to the 3D7 strain, identified proteins from KIL9605 isolate had a predicted median molecular weight of 53.0 KDa (range: 11.1-370.2) and a predicted median isoelectric point of 6.44 (range: 4.13-11.22). Searching the SignalP and TMHMM

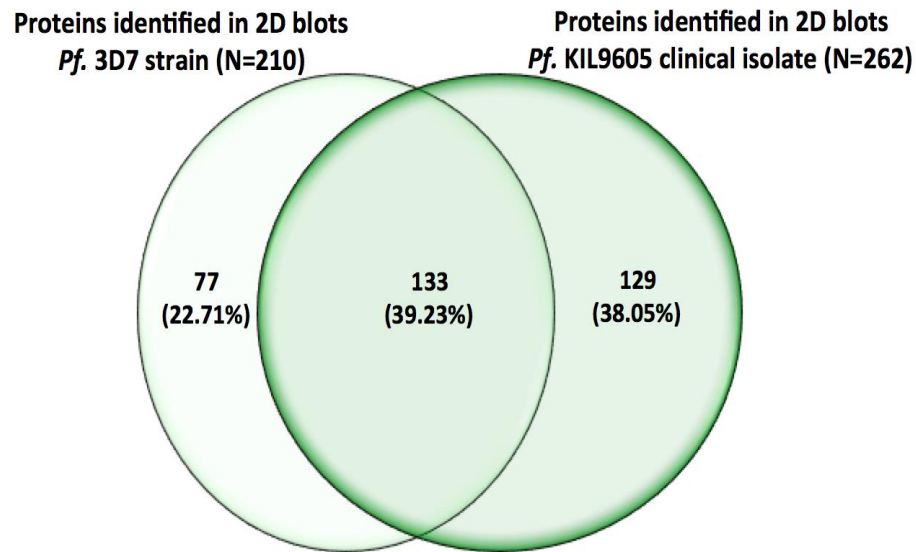
webservers revealed that forty-two (16.03%) and 37 (14.1%) of the identified proteins were predicted to have a signal peptide and transmembrane domain(s) respectively. The proportion of proteins with transmembrane domains identified was statistically lower than their occurrence within protein coding genes in the 3D7 genome (genome versus 2D-blot: 30.6% versus 14.1%;  $p\text{-value}<0.0001$ ). No differences were observed with proteins predicted to have a signal peptide (genome versus 2D-blot: 19.3% versus 16.0%;  $p\text{-value}=0.1891$ ). Of those proteins with predicted transmembrane domains, 30 (11.45%), 2 (0.76%) were predicted to have one and two transmembrane domains. One antigen (0.38%) was predicted to have three, four, six, seven and eleven transmembrane domains. I used transcriptome data available on PlasmoDB where the maximal transcriptional timing across the 48-hour asexual cycle is available. Similar to the results with the 3D7 strain, the median maximal transcription hours post invasion (hpi) was 30 hours. Proteins were classified as having their maximal transcription between 1-16 hpi: 57 (21.03%), 17-34 hpi: 95 (35.06%) and 35-48 hpi: 101 (37.27%) with the majority (72.3%) of identified proteins showing elevated transcription levels in the 17-34 hpi and 35-48 hpi. Transcription profile over the 48-hour asexual life cycle was not available for 18 (6.64%) of the identified antigens.

#### ***2.5.11. Comparison between antigens identified in the 2DE-Western Blot from 3D7 strain and KIL9605 isolate***

I had hypothesised that the antigenic determinants would vary between a recently lab-adapted clinical isolate obtained from a cerebral malaria patient and the lab-adapted 3D7 strain that has been maintained in *in vitro* culture in the absence of the host's immune system. I therefore compared the number of targets and their level of polymorphism between targets identified in the two parasite lines.

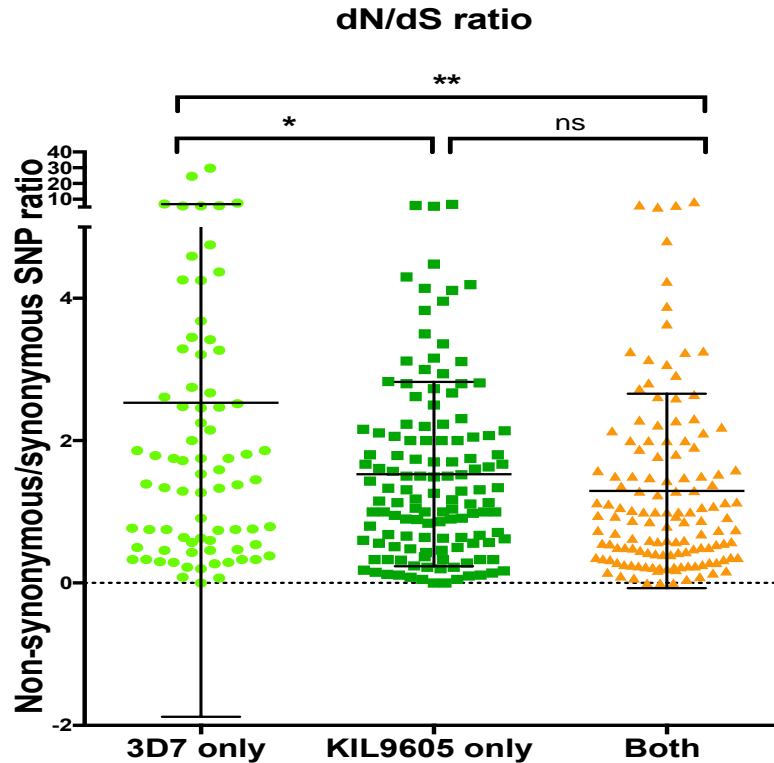
More proteins were identified by MIG using the KIL9605 isolate compared to the 3D7 strain (210 versus 262 proteins respectively). As shown in Figure 2.13, 133 (39%) proteins were identified in both sets of experiments while 77 (23%) and 129 (38.1%) were targets identified only with the 3D7 and KIL9605 isolate respectively.

Interestingly, targets found exclusively in the 3D7 2DE-blot had a higher level of polymorphisms compared to those found in the KIL9605 strain or both experiments. As shown in Figure 2.14, the ratio of non-synonymous /synonymous single nucleotide polymorphisms (dN/dS SNP ratio) was higher in the genes encoding antigens identified in the 3D7 blots (mean dN/dS SNP ratio in 3D7 versus KIL9605: 2.533 versus 1.529; p-value=0.0165, unpaired t-test).



**Figure 2.13: Comparison of the number of antigens identified with the 3D7 and KIL9605 isolate.** Venn diagram showing the overlap between proteins identified by mass-spectrometry in the 2D-blot from merozoite proteins extracted from 3D7 strain and KIL9605 clinical isolate.

There were no significant differences in the mean dN/dS ratio between genes encoding antigens identified exclusively in the KIL9605 strain compared to those found in both data sets. Lastly, the mean dN/dS ratio was higher in the 3D7 dataset compared to antigens identified in both data sets (mean dN/dS SNP ratio in 3D7 versus both: 2.533 versus 1.293; p-value=0.003, unpaired t-test).



**Figure 2.14: Comparing the level of polymorphism in antigens identified using the 3D7 or KIL9605 isolate.** The ratio of non-synonymous/synonymous SNPs (dN/dS) in the genes encoding proteins identified by mass-spectrometry exclusively in the 2D-blot from merozoite proteins extracted from 3D7 strain and KIL9605 clinical isolate were compared using the Mann-Whitney U test. \*: p-value <0.05, \*\*: p-value <0.001

The following arguments represent plausible explanations for the differences observed between targets identified in the KIL9605 isolate and the 3D7 strain. The higher proportion of antigens identified exclusively in the KIL9605 dataset could represent: 1) identification of immunogenic targets lost following spontaneous deletions of segments of chromosomes in 3D7, resulting in a lower hit rate with the 3D7 strain; 2) identification of immunogenic targets expressed in clinical isolates that serve as an immune evasion mechanism, by being a smoke screen that diverts antibody responses from “true” targets, expression of which are lost during long-term *in vitro* culture in the absence of host immunity; 3) the similarities between the KIL9605 isolate and the Malawi isolates circulating at the time immunoglobulins were obtained from malaria-immune adults were higher than those between the 3D7 strain and Malawi isolates. Of note, Malawi and Kenya are in East Africa while 3D7 strain originated from West Africa. Differences between isolates from difference

geographical regions have been reported at several genetic loci [332-334]. Interestingly, I show that genes identified exclusively in the 3D7 dataset were significantly higher in their level of non-synonymous polymorphisms when compared to targets identified in both datasets. Encouragingly, this may indicate the presence of immunogenic targets of limited polymorphisms or with conserved epitopes that are found across isolates from different geographical regions and was therefore identified as immunogenic in both strains. The targets identified exclusively in the 3D7 isolate were highly polymorphic, it is therefore conceivable that they may induce strong strain specific antibodies such that the antibodies obtained from Malawian adults would not recognise the variant antigen expressed in a Kilifi isolate.

Of the 339 proteins identified in the spots excised from 2DE-blot from both isolates, I found that a total of 59 (17%) proteins have been previously shown to be immunogenic. 26 were identified in both parasite isolates (Table 2.4) while additional 14 (Table 2.5) and 19 targets (Table 2.6) were identified in only the 3D7 strain and KIL9605 2DE-blot respectively. Although I replicated the 2D-gel and western blotting steps for both the 3D7 and KIL9605 isolate and observed the spot pattern for recognition was similar, I excised spots from a single gel representing one biological replicate for each isolate. As such, the differences observed between the isolates could be due to primarily technical factors. Establishing that these are real biological differences would require confirmation with additional replicate data from mass-spectrometry. Despite this limitation, the majority of targets identified, 280 (83%) had not been evaluated as targets of naturally acquired antibodies.



**Table 2.4: Table showing proteins identified in the 2DE-blot from both 3D7 and KIL9605 and previously shown to be immunogenic.**

Known immunogenic antigens identified in both 3D7 strain and KIL9605 isolate			
PlasmoDB identifier	Product description	Predicted signal peptide	Predicted transmembrane domains
PF3D7_0207600	Serine repeat antigen 5 (SERA5)	Yes	No
PF3D7_0207700	Serine repeat antigen 4 (SERA4)	Yes	No
PF3D7_0401800	Plasmodium exported protein (PHISTb), unknown function (PfD80)	No	Yes
PF3D7_0532100	Early transcribed membrane protein 5 (ETRAMP5)	Yes	Yes
PF3D7_0702400	Small exported membrane protein 1 (SEMP1)	No	Yes
PF3D7_0801000	Plasmodium exported protein (PHISTc), unknown function	No	Yes
PF3D7_0818200	14-3-3 protein (14-3-3I)	No	No
PF3D7_0818900	Heat shock protein 70 (HSP70)	No	No
PF3D7_0905400	High molecular weight rhoptry protein 3 (RhopH3)	Yes	No
PF3D7_0930300	Merozoite surface protein 1 (MSP1)	Yes	Yes
PF3D7_1008700	Tubulin beta chain	No	No
PF3D7_1014100	Conserved Plasmodium protein, unknown function	Yes	No
PF3D7_1028700	Merozoite TRAP-like protein (MTRAP)	Yes	Yes
PF3D7_1035400	Merozoite surface protein 3 (MSP3)	Yes	No
PF3D7_1115400	Cysteine proteinase falcipain 3 (FP3)	No	Yes
PF3D7_1129100	Parasitophorous vacuolar protein 1 (PV1)	Yes	No
PF3D7_1133400	Apical membrane antigen 1 (AMA1)	Yes	Yes
PF3D7_1149200	Ring-infected erythrocyte surface antigen	No	No
PF3D7_1222300	Endoplasmin, putative (GRP94)	Yes	No
PF3D7_1232100	60 kDa chaperonin (CPN60)	Yes	Yes
PF3D7_1311800	M1-family alanyl aminopeptidase (M1AAP)	No	Yes
PF3D7_1335100	Merozoite surface protein 7 (MSP7)	Yes	No
PF3D7_1401400	Early transcribed membrane protein 14.1 (ETRAMP14)	Yes	Yes
PF3D7_1436300	Translocon component PTEX150 (PTEX150)	Yes	No
PF3D7_1467900	Rab GTPase activator, putative	No	No

**Table 2.5: Table showing proteins identified in the 2DE-blot from 3D7 strain and previously shown to be immunogenic**

Known immunogenic antigens identified in the 3D7 strain 2DE-blot			
PlasmoDB identifier	Product description	Predicted signal peptide	Predicted TMDs
PF3D7_0206800	Merozoite surface protein 2 (MSP2)	No	No
PF3D7_0500800	Mature parasite-infected erythrocyte surface antigen, erythrocyte membrane protein 2 (MESA)	No	No
PF3D7_0532400	Lysine-rich membrane-associated PHISTb protein (LyMP)	No	Yes
PF3D7_0828800	GPI-anchored micronemal antigen (GAMA)	Yes	Yes
PF3D7_0935900	Ring-exported protein 1 (REX1)	No	Yes
PF3D7_1035300	Glutamate-rich protein (GLURP)	Yes	No
PF3D7_1035500	Merozoite surface protein 6 (MSP6)	No	No
PF3D7_1121600	Circumsporozoite-related antigen, exported protein 1 (EXP1)	Yes	Yes
PF3D7_1149000	Antigen 332, DBL-like protein (Pf332)	No	No
PF3D7_1228600	Merozoite surface protein 9 (MSP9)	Yes	No
PF3D7_1320800	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	No	No
PF3D7_1414400	Serine/threonine protein phosphatase PP1 (PP1)	No	No
PF3D7_1420700	Surface protein P113	Yes	Yes
PF3D7_1468100	Conserved Plasmodium protein, unknown function	No	No

**TMDs: Transmembrane domains**

**Table 2.6: Table showing proteins identified in the 2DE-blot from KIL9605 isolate and previously shown to be immunogenic.**

Known immunogenic antigens identified in the KIL9605 strain 2DE-blot			
PlasmoDB identifier	Product description	Predicted signal peptide	Predicted TMDs
PF3D7_0214100	Protein transport protein SEC31 (SEC31)	No	No
PF3D7_0214900	Rhoptry neck protein 6 (RON6)	Yes	No
PF3D7_0404900	6-cysteine protein (P41)	Yes	No
PF3D7_0405900	Apical sushi protein (ASP)	Yes	Yes
PF3D7_0407800	Conserved Plasmodium protein, unknown function	No	No
PF3D7_0501100.1	Heat shock protein 40, type II (HSP40)	No	Yes
PF3D7_0532300	Plasmodium exported protein (PHISTb), unknown function	No	No
PF3D7_0730300	Transcription factor with AP2 domain(s) (AP2-L)	No	No
PF3D7_0806800	Vacuolar proton translocating ATPase subunit A, putative	No	Yes
PF3D7_0918900	Gamma-glutamylcysteine synthetase (gammaGCS)	No	No
PF3D7_0922100	Ubiquitin-like protein, putative	No	Yes
PF3D7_1003800	U5 small nuclear ribonuclear protein, putative	No	No
PF3D7_1035700	Duffy binding-like merozoite surface protein (DBLMSP1)	Yes	No
PF3D7_1123400	Translation elongation factor EF-1, subunit alpha, putative	No	No
PF3D7_1252100	Rhoptry neck protein 3 (RON3)	Yes	Yes
PF3D7_1335400	Reticulocyte binding protein 2 homologue a (RH2a)	No	Yes

### 2.5.12. Novel immunogenic merozoite antigens

The majority of targets I identified in either the 3D7 or KIL9605 isolate had not been evaluated in the context of naturally acquired immunity. I analysed these targets further in the following ways. First, I investigated the overlap between the potentially novel targets (N=280) with a network of genes proposed to play a role in erythrocyte invasion by merozoites, based primarily on concurrent transcription on exposure to parasite growth inhibitory compounds [258]. Secondly, I compared the identified targets with those identified to be under balancing selection in a previous whole genome sequence study of 65 clinical isolates from the Gambia [253]. Genes under balancing selection are thought to be exposed to immune pressure in order to maintain different alleles in the population at intermediate frequencies [335]. Lastly, I tested for the overlap of the targets I identified with genes where genetic manipulation of rodent malaria orthologues (primarily *P. berghei*) had been attempted as of October 2016, using data deposited in these two databases: i) RMgmDB: (<http://www.pberghei.eu/index.php>) and ii) PlasmoGEM: (<http://plasmogem.sanger.ac.uk/>) [336]. These may provide valuable information on genes that may be essential for parasite growth during the asexual blood-stages of the life cycle. As shown in Table 2.7, genes termed as invasion-related or under-balancing selection, were significantly higher in proportion within this 2D dataset than their occurrence within the genome. In contrast, *P. berghei* orthologues whose genetic modification classified the genes as either essential, resulted in reduced growth rate or dispensable over the asexual growth cycle, were significantly lower in proportion within the 2D-blot datasets obtained in my study. This may suggest that *P. falciparum* parasites may have evolved ways to shield proteins that are essential for growth and survival from protective antibody responses.

Of the potential novel targets (N=280), 30 (11%) are considered to play important roles in erythrocyte invasion and 19 (7%) are considered to be under balancing selection as listed in Table 2.7 and 2.8 respectively. Attempts to genetically disrupt their rodent malaria orthologous have been reported for 49 (18%) and 162 (58%) of the novel targets identified on the RMgmDB and PlasmoGEM databases

respectively. Of these targets, 14 (Table 2.9) and 109 (Appendix 2.3) may represent essential blood-stage antigens as the disruption of their *P. berghei* orthologues have been attempted but were not successful. Several antigens I identified in this study were amenable to gene disruption (35 and 53 in the RMgmDB and PlasmoGEM databases, respectively). Interestingly, disruption of some of these genes showed significant differences in their asexual blood-stage growth rate when compared to wild-type parasites.

In the context of naturally acquired immunity, antigens of interest for further evaluation would include: i) identified as immunogenic in my study but have not been evaluated as such ii) proposed to play a role in erythrocyte invasion (invadome) and iii) may play an essential role over asexual stage of the life cycle. For example, PF3D7\_1455300, a target described as a “conserved *Plasmodium* protein, unknown function” on PlasmoDB, has been proposed to be a member of the core “invadome” [258] and shown to be under balancing selection (Tajima  $D=0.015$ ) [253]. The *P. berghei* orthologue (PBANKA-1319000) is termed as an essential gene over the asexual stages of the life cycle in both the RMgmDB and PlasmoGEM databases. Thirteen targets shared similar features described above representing potential important targets of naturally acquired immunity whose evaluation and prioritization is warranted.

**Table 2.7:** Table showing a comparison between the occurrences of proteins of specific characteristics within the 2DE-blot data set and the genome.

Feature	Proportion found within the genome# N=5305 (%)	Proportion found within entire <sup>a</sup> and novel <sup>b</sup> dataset	P-value
Member of the invadome	418 (7.88)	a: 50 (14.8) b: 30 (11.0)	<b>0.0025</b> 0.0613
Positive Tajima D*	337 (6.7)	a: 35 (10.3) b: 19 (7.0)	<b>0.0115</b> 0.8452
<i>P. berghei</i> phenotype suggests the genes play essential functions over asexual stages <sup>%</sup>	1195 (46.4)	a: 125 (36.9) b: 109 (38.9)	<b>0.0010</b> <b>0.0167</b>
<i>P. berghei</i> phenotype suggests modification of the gene results in a reduced growth rate over asexual stages <sup>%</sup>	456 (17.7)	a: 34 (10.0) b: 28 (10.0)	<b>0.0004</b> <b>0.0011</b>
<i>P. berghei</i> phenotype suggests the genes play dispensable roles over asexual stages <sup>%</sup>	911 (35.4)	a: 28 (8.3) b: 25 (8.9)	<b>&lt;0.0001</b> <b>&lt;0.0001</b>

#- Protein coding genes (excluding pseudogenes) on PlasmoDB. \*-N=5056 genes were analysed. %- N=2575 number of genes with a phenotype reported on PlasmoGEM. a:-N=339 and b:-N=280

**Table 2.8: Novel Nineteen (19) *P. falciparum* antigens identified as being under balancing selection.**

<i>Plasmodium falciparum</i> gene ID	Product description	Tajima D scores	Additional notes
PF3D7_0202100	liver stage associated protein 2 (LSAP2)	0.208	Proposed to be essential for invasion
PF3D7_0318200	DNA-directed RNA polymerase II subunit RPB1 (RPB1)	0.72	
PF3D7_0424600	Plasmodium exported protein (PHISTb), unknown function	2.298	
PF3D7_0508800	single-stranded DNA-binding protein (SSB)	1.789	
PF3D7_0627800	acetyl-CoA synthetase, putative (ACS)	0.908	
PF3D7_0806500	DnaJ protein, putative	0.233	
PF3D7_0807800	26S proteasome regulatory subunit RPN10, putative (RPN10)	0.216	
PF3D7_0808300	ubiquitin regulatory protein, putative	0.062	Proposed to be essential for invasion
PF3D7_0810800	Hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase (PPPK-DHPS)	0.155	
PF3D7_0831400	Plasmodium exported protein, unknown function	0.132	
PF3D7_1115300	cysteine proteinase falcipain 2b (FP2B)	0.062	
PF3D7_1134000	heat shock protein 70 (HSP70-3)	1.301	
PF3D7_1229500	T-complex protein 1 subunit gamma (CCT3)	2.016	
PF3D7_1343700	kelch protein K13 (K13)	0.084	
PF3D7_1347700	ethanolamine-phosphate cytidyltransferase (ECT)	0.581	Mutant parasites showed a reduced asexual blood-stage growth rate when compared to wild-type parasites.
PF3D7_1407800	plasmepsin IV (PM4)	0.656	Mutant parasites showed a slightly reduced growth rate and a virulence-attenuated phenotype in mice when compared to wild-type parasites[337].
PF3D7_1428300	proliferation-associated protein 2g4, putative	0.777	
PF3D7_1430300	acid phosphatase, putative	0.43	
PF3D7_1455300	conserved Plasmodium protein, unknown function	0.015	Proposed to be essential for invasion. Two attempts to disrupt the gene were not successful.

**Table 2.9: Forty-nine *P. falciparum* genes in which genetic disruption their rodent malaria orthologues have been attempted**

<i>Plasmodium falciparum</i> gene ID	Product description	Proposed member of invadome	Positive Tajima D	RMgmID	Rodent malaria gene ID	Modification attempted in rodent malaria parasites
<b>Efforts to disrupt or knock-down the gene was not successful</b>						
PF3D7_0305300	Conserved Plasmodium membrane protein, unknown function		0	RMgm-244	PBANKA_0403800	Two attempts to disrupt the gene were not successful*
PF3D7_0422300	alpha tubulin 2		0	RMgm-4	PBANKA_0522700	Five attempts to disrupt the gene were not successful[338].
PF3D7_0503400	actin-depolymerizing factor 1 (ADF1)		0	RMgm-388	PBANKA_1103100	Four attempts to disrupt the gene were not successful[339].
PF3D7_0714000	histone H2B variant (H2B.Z)		0	RMgm-3838	PBANKA_1420600	Attempts to disrupt the gene were not successful*. Numbers of attempts were not reported.
PF3D7_0919000	nucleosome assembly protein (NAPS)		0	RMgm-217	PBANKA_0819900	Four attempts to genetically disrupt the gene were not successful. Very small proportion of mature-schizonts showed GFP expression[340].
PF3D7_1023900	chromodomain-helicase-DNA-binding protein 1 homolog, putative (CHD1)		0	RMgm-507	PBANKA_0508100	Two attempts to disrupt the gene were not successful.
PF3D7_1025300	conserved Plasmodium protein, unknown function		0	RMgm-80	PBANKA_0509500	Three attempts to disrupt the gene were not successful[341].
PF3D7_1145400	dynammin-like protein (DYN1)		0	RMgm-238	PBANKA_0903600	Three attempts to disrupt the gene were not successful*.
PF3D7_1204300	eukaryotic translation initiation factor 5A (EIF5A)		0	RMgm-1580	PBANKA_0603200	Two attempts to disrupt the gene were not successful[342].
PF3D7_1316600	choline-phosphate cytidylyltransferase (CCT)		0	RMgm-335	PBANKA_1415100	Two attempts to disrupt the gene were not successful[343].
PF3D7_1324900	L-lactate dehydrogenase (LDH)		0	RMgm-3592	PBANKA_1340100	Attempts to disrupt the gene were not successful
PF3D7_1360800	falcilysin (FLN)		0	RMgm-804	PBANKA_1137000	Three attempts to disrupt the gene were not successful.
PF3D7_1455300	conserved Plasmodium protein, unknown function	1	1	RMgm-509	PBANKA_1319000	Two attempts to disrupt the gene were not successful.
PF3D7_1454700	6-phosphogluconate dehydrogenase, decarboxylating, putative		0	RMgm-3494	PBANKA_1318400	Attempts to disrupt the gene were not successful.
<b>Efforts to disrupt or knock-down the gene was successful</b>						
PF3D7_0309500	Asparagine synthetase, putative		0	RMgm-1872	PBANKA_0407600	Gene disruption successful. Significantly reduced growth rate when compared to wild-type parasites. Reduced ookinete, oocyst and sporozoite production[344].
PF3D7_0501600	rhoptry-associated protein 2 (RAP2)	1	0	RMgm-209	PBANKA_1101400	GFP-tagged RAP2 showed expression in maturing merozoites within schizonts and a two-dot staining[345]. Disrupted gene showed significantly reduced growth rate when compared to wild-type parasites.
PF3D7_0502400	ring-stage membrane protein 1, merozoite surface protein 8 (MSP8)		0	RMgm-225	PBANKA_1102200	<i>P. berghei</i> parasites expressing MSP8 lacking the GPI-anchor and C-terminal EGF domains showed no difference in growth rate when compared to wild-type[346].
PF3D7_0507500	subtilisin-like protease 1 (SUB1)	1	0	RMgm-972	PBANKA_1107100	HA-tagged PbSUB1 showed expression in blood and liver stages. Plays essential role in merozoite egress from schizonts and parasite egress from hepatocytes[347, 348].

<i>Plasmodium falciparum</i> gene ID	Product description	Invadome	Positive Tajima D	RMgmID	Rodent malaria gene ID	Modification attempted in rodent malaria parasites
PF3D7_0608800	ornithine aminotransferase (OAT)		0	RMgm-1568 RMgm-1642	PY17X_0109000  PBANKA_0107400	GFP tagged <i>P. yoelii</i> showed internal localization.  Genetic disruption of <i>P. berghei</i> gene showed no difference in asexual growth rate when compared to wild type.
PF3D7_0903700	alpha tubulin 1		0	RMgm-1925	PBANKA_0417700	Gene disruption successful. No difference in growth rate between mutant and wild-type parasites.
PF3D7_0904900	copper-transporting ATPase (CuTP)		0	RMgm-960/1	PBANKA_0416500	Gene disruption successful. No differences observed in asexual blood-stages. Ookinete, oocyst and sporozoite formation reduced[349].
PF3D7_1003600	inner membrane complex protein 1c, putative (IMC1c)	1	0	RMgm-1122	PBANKA_1202000	Significantly reduced growth rate in mutant parasites when compared to wild-type parasites. mCherry tagged <i>PbIMC1c</i> showed localization to the periphery of individual merozoites[350].
PF3D7_1021600	deoxyribose-phosphate aldolase, putative		0	RMgm-1553	PY17X_0506900	<i>PyGFP</i> -tagged parasites showed localization to the cytoplasm.
PF3D7_1033400	haloacid dehalogenase-like hydrolase (HAD1)		0		PY17X_0518300	Gene disruption was successful. No differences observed between asexual blood stage growth rate in mutant and wild-type parasites.
PF3D7_1104000	phenylalanine--tRNA ligase beta subunit		0	RMgm-2690	PBANKA_0942900	Genetic disruption was successful. Growth rate phenotype has not been described.
PF3D7_1113700	glyoxalase I (GloI)		0	RMgm-2649	PBANKA_0933900	Gene disruption was successful. No differences observed between asexual blood stage growth rate in mutant and wild-type parasites.
PF3D7_1115600	peptidyl-prolyl cis-trans isomerase (CYP19B)		0	RMgm-253	PBANKA_0932200	Gene disruption was successful. Growth rate phenotype has not been described.
PF3D7_1116700	cathepsin C, homolog, dipeptidyl aminopeptidase 1 (DPAP1)		0	RMgm-810	PBANKA_0931300	Gene disruption was successful. Reduced asexual blood-stage growth rate in mutant compared to wild-type parasites.
PF3D7_1124600	ethanolamine kinase (EK)		0	RMgm-2590	PBANKA_0923700	Gene disruption was successful. Reduced asexual blood-stage growth rate in mutant compared to wild-type parasites.
PF3D7_1130200	60S ribosomal protein P0 (Pfp0)		0	RMgm-1561	PY17X_0919700	GFP-tagged <i>Py</i> parasites showed localization in the cytoplasm of asexual stages
PF3D7_1135100	protein phosphatase 2C, putative		0	RMgm-1046	PBANKA_0913400	<i>PbΔppm8</i> mutant parasites showed no differences from wild-type parasites in the asexual, sexual and liver stages.
PF3D7_1138500	protein phosphatase 2C (PPM2)		0	RMgm-1041	PBANKA_0910200	<i>PbΔppm2</i> mutant parasites showed no differences from wild-type parasites in the asexual blood stages. However, reduced numbers of macrogametes were observed and ookinete differentiation at stage II observed[351].
PF3D7_1222700	glideosome-associated protein 45 (GAP45)	1	0	RMgm-776	PBANKA_1437600	GAP45 is considered vital for merozoite invasion of erythrocytes. <i>PbΔGAP45</i> zygotes failed to develop into ookinetes[352].
PF3D7_1229400	macrophage migration inhibitory factor (MIF)		0	RMgm-26	PBANKA_1444000	<i>Pbmif</i> -ko showed no differences in entire life-cycle growth rate or virulence. Rodent hosts infected with mutant parasite showed higher number of reticulocytes[353].
PF3D7_1235700	ATP synthase subunit beta, mitochondrial		0	RMgm-4000	PBANKA_1450300	Mutant parasites showed reduced asexual growth rate when compared to wild-type parasites.
PF3D7_1246200	actin I (ACT1)	1	0	RMgm-678	PBANKA_1459300	GFP-tagging showed expression in all asexual blood stages, ookinetes, oocysts, sporozoites and liver stages[354].



<i>Plasmodium falciparum</i> gene ID	Product description	Invadome	Positive Tajima D	RMgmID	Rodent malaria gene ID	Modification attempted in rodent malaria parasites
PF3D7_1251200	coronin	1	0	RMgm-1497	PBANKA_1464100	<i>Pb</i> coronin- parasites showed reduced numbers of sporozoites in mosquito salivary glands, reduced liver stage development[355].
PF3D7_1342600	myosin A (MyoA)	1	0	RMgm-656	PBANKA_1355700	<i>Pb</i> mutant parasites showed no difference in asexual and gametes compared to wild-type. Ookinete motility was reduced and oocyst production was reduced[352].
PF3D7_1347500	DNA/RNA-binding protein Alba 4 (ALBA4)		0	RMgm-3699	PBANKA_1360300	Mutant parasites showed a reduced asexual blood-stage growth rate when compared to wild-type parasites.
PF3D7_1347700	ethanolamine-phosphate cytidyltransferase (ECT)		1	RMgm-3701	PBANKA_1360500	Mutant parasites showed a reduced asexual blood-stage growth rate when compared to wild-type parasites.
PF3D7_1361800	conserved Plasmodium protein, unknown function	1	0	RMgm-710/725	PBANKA_1137800	mCherry tagged parasites showed localization to the cytoplasm of parasites.
PF3D7_1404900	conserved Plasmodium protein, unknown function		0	RMgm-1482	PBANKA_1037300	GFP-tagged parasites generated. No phenotype has been described.
PF3D7_1407800	plasmepsin IV (PM4)		1	RMgm-316	PBANKA_1034400	Mutant parasites showed a slightly reduced growth rate and a virulence-attenuated phenotype in mice when compared to wild-type parasites[337].
PF3D7_1412500	actin II (ACT2)		0	RMgm-632/633	PBANKA_1030100	<i>PbactII</i> - showed no differences when compared to wild type, however reduced exflagellation of male gametocytes and reduced ookinete and oocyst formation[356].
PF3D7_1438900	thioredoxin peroxidase 1 (Trx-Px1)		0	RMgm-206	PBANKA_1302800	<i>PbPrx</i> -KO showed no differences from wild-type parasites, however reduced production of gametocytes were observed[357, 358]. Reduced numbers of liver stage merozoites[359].
PF3D7_1446200	M17 leucyl aminopeptidase (LAP)		0	RMgm-814	PBANKA_1309900	<i>Pb</i> Δlap mutant parasites showed a strongly reduced growth rate of blood stages when compared to wild-type parasites.
PF3D7_1454400	aminopeptidase P (APP)		0	RMgm-813	PBANKA_1318100	<i>Pb</i> Δaapa/b mutant parasites showed a reduced growth rate in mice and produces reduced levels of haemozoin when compared to wild type parasites.
PF3D7_1460600	inner membrane complex sub-compartment protein 3, putative (ISP3)	1	0	RMgm-962/964	PBANKA_1324300	<i>Pb</i> Δisp3 showed no differences in asexual blood-stage when compared to wild type parasites. GFP-tagged parasites showed localization in schizonts and merozoites[360].
PF3D7_1466100	protein phosphatase containing kelch-like domains (PPKL)		0	RMgm-785	PBANKA_1329500	<i>Pb</i> ppkl- mutant parasites showed no difference in asexual blood stages however, aberrant developments of ookinetes were observed with reduced motility. No oocysts and sporozoites were formed in mutant parasites[361].

## 2.6. Discussion

### ***Proteomic-serology approaches for the discovery of immunogenic merozoite antigens***

Comprehensive screening approaches such as the proteomic methods employed in this study have the potential to describe a greater range of specificities of immune responses to complex pathogens. Immunoprecipitation techniques using pathogen lysate material have been used widely to identify targets and protein-protein interacting partners. On the other hand, although it is labour-intensive, 2DE gels coupled to serology has been used to answer a range of biomedical research questions such as the identification of the targets of ‘self-antigen’ recognizing antibodies in autoimmune diseases, biomarker discovery or for vaccine discovery in infectious diseases [313]. For instance, the approach has been used to identify the targets of self-antibodies in autoimmune diseases such as the rare inflammatory Behcet’s disease [362-364] that results in damage to blood vessels, membranous glomerulonephritis that affects the kidneys[365] and type 1 diabetes [366]. Similarly, it has been successfully used to identify potential vaccine candidates for human diseases such as the bacterial infection by *Chlamydia pneumonia*[367] and *Bordetella pertussis* [368], or the parasitic infection by *Leishmania donovani*[369].

### ***Identifying immunogenic *P. falciparum* merozoite antigens: quality of reagents***

Mining data from PlasmoDB indicated that there were many *P. falciparum* merozoite antigens that were potentially immunogenic (639 potential targets) but had not been evaluated in this regard (see section 2.5.1). These could represent novel targets that elicit antibody responses that play a role in halting the erythrocytic life cycle of the malaria parasite. Therefore, I employed the two proteome-serology approaches to identify the targets of naturally acquired antibodies to merozoite antigens that could help prioritize a set of potential vaccine candidates. To confirm that my reagents were of high quality, I conducted a series of tests. First, I ensured that my method of merozoite extraction (adapted from Boyle *et al*, 2013) [370] yielded high quality merozoite preparations in multiple ways. I confirmed the presence of known merozoite antigens that vary in their function and localization.

These included peripherally associated surface protein MSP3, an erythrocyte invasion ligand located in the micronemes before invasion of erythrocytes, EBA175, members of the glideosome complex involved in driving erythrocyte invasion and located between the plasma membrane and the underlying inner membrane complex (IMC) such as GAP45, GAP50, MyoA and MTIP [326]. Secondly, I ensured the pool of negative sera was obtained from individuals with no previous exposure to *P. falciparum* malaria by confirming non-reactivity as measured by ELISA to the immunodominant merozoite antigen AMA1 and total *P. falciparum* schizont extract. Lastly, I confirmed by electron microscopy that the purified merozoites obtained following *in vitro* culture contained antigenic determinants. These were preferentially detected by the pool of purified immunoglobulins from malaria-immune adults (MIG), and were primarily localised on the surface of the merozoites. The MIG preparation had been previously shown to bind to parasite derived surface antigens on erythrocytes in twenty-two clinical isolates from Malawi [328]. They further ascertained that the preparation contained antibodies with different specificities as the eluted antibodies, following adsorption with one isolate, recognised the homologous isolate but not heterologous ones. Lastly, the immunoglobulin preparation was also shown to be able to reverse the binding between infected erythrocytes and melanoma cells in an *in vitro* cyto-adherence reversal assay [328]. These observations provided the rationale for using MIG as a test immunoglobulin preparation whose target antigens would be of interest to identify.

### ***Identifying immunogenic merozoite antigens by 2DE western blots***

I coupled 2-dimensional gel electrophoresis (2DE) to western blotting using MIG to identify reactogenic *P. falciparum* merozoite antigens. This approach has been used in various infectious diseases to identify immunogenic targets. For example, thirty immunoreactive antigens against the causative agent of whooping cough, *Bordetella pertussis*, were identified using sera from ten children vaccinated with whole-cell pertussis vaccine (WCV) [368]. This represents 1% of the predicted protein coding genes (3456 genes) [371] in the entire *B. pertussis* genome. Similarly, the protozoan

*Leishmania donovani* that causes leishmaniasis has also been evaluated for antigenicity by 2DE-blot [369]. In this study, 330 immunogenic spots were observed representing 4% of the protein coding genes in the *L. donovani* genome (8336) [372]. Using a similar approach, I identified 210 and 262 putative immunogenic merozoite antigens from the lab-adapted *Pf3D7* strain and a recently isolated and lab-adapted Kenyan clinical isolate respectively. This corresponds to only 3.8% and 4.7% of the entire protein coding genes in the *P. falciparum* genome. This particular approach has not been employed in the analysis of immunogenic *P. falciparum* antigens from any stages of the lifecycle, which has limited comparisons.

### ***Novel immunogenic P. falciparum targets***

I compared the targets I identified with published literature, and found fifty-nine antigens (17%) that have been previously shown to be immunogenic primarily through screening recombinant antigens by protein arrays or ELISA using sera from individuals with varying degrees of exposure and susceptibility to *P. falciparum* malaria [146, 147, 263, 265, 267, 292]. These include known merozoite antigens that are currently under evaluation as blood-stage vaccines AMA1, GLURP, MSP1, 2, 3 and SERA 5 [305, 373, 374]. In addition, antigens that have been analysed more recently such as the peripherally associated merozoite surface antigens MSP 6, 7, 9 [146, 147] and MSPDBL1 [292], cysteine proteases released following merozoite egress such as SERA4 and SERA5 [146], rhoptry neck proteins e.g. RON3 [267], the high molecular weight rhoptry proteins localised in the rhoptry bulbs e.g. RhopH3 [267] and the GPI-anchored Micronemal Antigen, GAMA [146]. The identification of known immunogenic merozoite antigens gave me the confidence that this approach could lead to the discovery of novel antigens. There were merozoite antigens that are known to be targets of naturally acquired antibodies but were not identified in the 2D-blot experiments. These included MSP4, MSP5, MSP10, DBLMSP2, EBA-181 and EBA-175 [146, 147, 292]. These targets may have been missed due to the inability of 2D-blot to enrich for

hydrophobic or membrane proteins as is discussed in detail in the study limitations section on page 130.

Interestingly, a large proportion of identified targets (83%) had not been evaluated in the context of naturally acquired immunity, representing potential novel antigens that should be analyzed further and evaluated as targets of protective antibodies. Encouragingly, some of these targets have been proposed to play important roles such as in erythrocyte invasion, or show interesting signatures such as evidence of balancing selection or a phenotype following genetic modification in several studies. Targets such as the conserved protein, PF3D7\_1455300, has been proposed to be a member of the invadome and the attempts to disrupt the function of the *P. berghei* orthologue have been unsuccessful. This implies that the target plays an essential role over the asexual stage of the life cycle. It is therefore plausible that antibodies generated against this target may inhibit or halt parasite growth altogether warranting further evaluation of these targets as well as thirteen other targets that share similar features.

#### ***Differences between immunogenic targets identified using a lab-adapted and clinical P. falciparum isolate***

I had hypothesised that the antigenic determinants would vary between merozoite antigens obtained from a lab-adapted parasite line (3D7) that was first adapted to *in vitro* culture in 1981 [375] and a clinical isolate from Kilifi, Kenya that was first isolated and put into *in vitro* culture on the 29<sup>th</sup> of July, 2009 (Abdirahman Abdi, personal communication). Differences between *P. falciparum* isolates have been documented before, that have had an effect on: i) parasite virulence or, ii) their susceptibility to drugs such as chloroquine [376] or iii) their ability to infect *Anopheles gambiae* mosquitoes [377] or iv) in their invasion phenotype and merozoite invasion ligand expression profiles [378]. In line with this hypothesis, we observed a higher number of antigens identified as immunogenic in the clinical isolate compared to the lab-adapted isolate. In addition, the targets identified in both

isolates were less polymorphic compared to targets identified exclusively in the 3D7 isolate. Additional replicate data are required to confirm these findings.

There are several plausible explanations for these differences that include: i) deletions of genomic regions or transcriptional repression that vary between isolates or ii) immune evasion mechanisms employed by different isolates or iii) similarities between circulating parasites from a similar geographical region. For example, 3D7 is known to delete sub-telomeric regions of its genome particularly on chromosome 2 and 9 following long-term culture [330, 331]. Deletions in the sub-telomeric regions of chromosome two results in the loss of KAHRP (PF3D7-0202000), a protein involved in parasite cytoadherence. Interestingly, the gene PF3D7-0202100 encoding liver stage associated protein 2 (LSAP2), which is adjacent to KAHRP, was identified as immunogenic exclusively in the clinical isolate's immunoblots. Similarly, some genes have been shown to vary in their mRNA abundance between isolates when compared by microarray suggesting strain specific transcription regulation. For example, in a microarray analysis that compared mRNA levels between the lab-adapted isolates 3D7, HB3 and Dd2, they identified genes whose transcripts were highly abundant in a strain specific manner such as MSP2 that was highly abundant in the 3D7 isolate [257]. Similarly, in my study, MSP2 was identified as immunogenic in the 3D7 immunoblots only. Lastly, the expression of merozoite invasion ligands, have been shown to vary between isolates from the same [379] or varied geographical regions [378]. Interestingly, the invasion ligand, Reticulocyte Binding Protein 2 Homologue a (RH2a), was identified in the clinical isolate only.

Although difficult to validate with the data available, it is plausible that the targets identified exclusively in the individual data sets could represent targets that serve as a “smoke screen” diverting antibody responses away from true targets. Encouragingly, I observed that targets identified in both isolates were less polymorphic compared to those identified in each of the isolates exclusively. This would suggest the presence of antigenic targets that are of limited polymorphism or

conserved and therefore likely to be immunogenic regardless of the geographical origin of the isolate. This has potential implications on vaccine design and efficacy. Previous work has shown that vaccine candidates based on highly polymorphic proteins such as MSP2 and CSP have elicited strain specific immunity [171, 222]. To circumvent this problem, approaches such as generation of a multi-valent vaccine (containing two or more alleles of the same antigen) that covers the majority of circulating alleles are being evaluated [380]. A second approach would be to focus on antigens that are targets of naturally acquired immunity and are of limited polymorphism as they are likely to be effective across diverse geographical regions [380].

### ***Study limitations***

I observed a lack of specificity and reproducibility in the pull-down assays I conducted, which is common in this assay. This is often attributed to various sources that include: i) non-specific binding of parasite proteins with a high-affinity to Protein G and/or ii) high affinity protein-protein interaction present in a target bound by antibodies that are co-eluted [381]. For instance, a human monoclonal antibody with broad agglutinating activity of infected erythrocytes pulled down several targets evidenced by multiple bands on a western blot and multiple hits on mass-spectrometry identification of eluates [382]. Similarly, rabbit anti-P12 sera immunoprecipitated six targets from *P. falciparum* schizont lysates and only one target (P41) was subsequently validated as the true interacting partner [383]. The specificity of pull-down assays can be improved using stringent washing steps or pre-incubating extracted parasite proteins with Protein G before proceeding with the immunoprecipitation. While immunoprecipitation of antigens from *P.falciparum* parasite lysate material has been successfully used to identify antigens such as AMA1 [244], MSP1 [241] and MSP2 [242], it is worth noting that these experiments used either a monoclonal antibody or polyclonal sera against a single antigen. The quantities of antigen-specific antibodies in polyclonal sera obtained from animal immunization ranges from 200µg/ml-3000µg/ml (www.agrisera.com). This would be considerably higher in concentration, affinity and avidity than the

antigen-specific IgG present in a pool of immunoglobulins from adults whose malarial specific responses vary considerably between individuals, in addition to being exposed to a range of other pathogens [384, 385]. These may explain why the signal by western blot from MIG eluates did not differ from non-exposed controls.

Although I have improved on the 2DE maps provided by Schmidt-Ullrich *et al* [317] and Gelhaus *et al* [315] whose maps were limited to a pH range of 4-7, I was limited to identifying immunogenic antigens whose isoelectric points lie between 3-10. A total of 743 *P. falciparum* genes are predicted to having an isoelectric point above 10 (<http://plasmodb.org/>) and would have been missed by this approach. Similarly, high molecular weight proteins above 200 kDa were unlikely to be identified due to the resolution limits of a 12% SDS-gel. Three hundred and forty five (345) *P. falciparum* protein-coding genes are predicted to have molecular weights above 250 kDa, and would also have been missed by this approach. In addition, a known limitation of isoelectric focusing in the first dimension is its inability to solubilise and focus hydrophobic proteins, particularly membrane proteins that tend to precipitate [314, 386, 387]. As a result, proteins containing transmembrane domains are under represented in my 2DE maps. This was evident with my data where only 18% of identified targets had a transmembrane domain; significantly lower than proportions observed within the genome (section 2.5.6 and 2.5.10). This is of particular concern given the observation that purified immunoglobulins from malaria-immune adults localised primarily on the surface of the merozoite as shown in section 2.5.3, suggesting they react to membrane-bound proteins. One possible solution that would aid in the enrichment and resolution of membrane proteins in a two-dimensional gel is the combination of a blue-native polyacrylamide gel electrophoresis (BN-PAGE) [388] coupled to SDS-PAGE. This technique differs with the method I used only at the protein extraction and separation in the first dimension. The blue-native polyacrylamide gel electrophoresis has the following modifications: i) proteins are extracted using non-ionic detergents such as digitonin, that prevents disruptions of protein-protein interactions but efficiently solubilises lipophilic interactions such as those between



integral membrane proteins and the lipid-bilayer. ii) non-denaturing conditions are maintained in which SDS or urea is not present in any buffers and 3) the addition of an anionic dye in the extraction buffer that imparts an overall negative charge to aid migration during electrophoresis. Lastly, an additional limitation is that proteins separated by 2DE lose their native confirmation due to reduction and denaturation (in the second dimension separation) and as result, antigens that solely rely on conformation dependent epitopes for antibody recognition may be under-represented.

In summary, despite the limitations highlighted above, coupling bi-dimensional gel electrophoresis to fluorescent western blots has identified potential novel targets of naturally acquired immunity. These data indicate that there are many more immunogenic antigens that have not been evaluated for their utility as vaccine candidates. This fits well with recently published work in which additional blood-stage antigens that have not been studied before were evaluated as potential vaccine candidates and out-performed some of the candidates currently in clinical trials [146, 147, 389, 390]. Of the immunogenic targets I identified, a large proportion of them have not been studied particularly in the context of immunity. Interestingly, some of these targets have been proposed to be members of the “invadome” that may play a key role in erythrocyte invasion by merozoites [258]. Other antigens have encouraging phenotypes when their orthologue in the rodent malaria *P. berghei* is genetically modified[391], either leading to reduced asexual growth, or a simply not able to be deleted at all, suggesting they may be essential for growth. The targets I identified are a rich resource that should be further evaluated and validated as targets of protective immunity. In subsequent chapters, I selected a subset of these antigens and show that they are immunogenic and elicit varying antibody responses in adults living in a malaria endemic region of Tanzania. Some of these targets elicited antibody responses that were associated with protective immunity to *P. falciparum* malaria. Details of the down-selection procedures of the subset of antigens for further analysis and their subsequent evaluation in immuno-epidemiological studies are presented in Chapter four and five of this thesis.

## CHAPTER 3

### **Describing the *Plasmodium falciparum* merozoite surface proteome using surface trypsinization and biotinylation**

#### **3.0. Introduction**

The majority of infectious agents interact with the extracellular environment and the host's immune system through their cell surface proteins [392]. Intracellular pathogens can use their surface proteins as ligands to bind to receptors on host cells thereby facilitating invasion [393], or as attachment and adherence factors during the colonization of tissues or organs [394], which can also enhance pathogenesis [395]. Alternatively, surface proteins may bind to soluble immune effector factors or cells to evade or modulate the hosts' protective defences [394, 396]. Lastly, surface proteins can play key roles in sensing the chemical or physical cues of their external environment, and in the uptake of nutrients and molecules that are essential for pathogen survival. Due to their vital roles in pathogen tropism, survival and their interaction with the host's immune defences, surface proteins often represent attractive drug and vaccine targets [395, 397].

Several vaccines that are either commercially available or under development are based on surface-exposed pathogen proteins. One example is the 4CMenB multicomponent vaccine developed against the bacteria *Neisseria meningitidis* serogroup B [398], which causes sepsis and meningitis in children and young adults and is often fatal. The 4CMenB vaccine is composed of three immunogenic antigens that are located on the surface of the bacteria namely *Neisseria* Adhesin A (NadA), *Neisseria* heparin binding antigen (NHBA) and factor H binding protein (fHbp) [399]. This multicomponent formulation induced antibodies in mice that were bactericidal against 35 (78%) of the tested meningococcal strains. Phase I, IIb and III clinical trials have been conducted showing immunogenicity in infants and adults, and which induced antibodies with bactericidal activity [398]. Similarly, a second vaccine candidate, Spy0416 antigen, is a membrane protein identified from surface shaving *Streptococcus* group A bacteria[400]. The recombinant form of the

identified antigen was subsequently shown to be protective in a mouse immunization, followed by challenge study where they used a virulent group A streptococcus bacteria [400].

In the context of *P. falciparum* malaria, the majority of the vaccine candidates in clinical trials target antigens that localize to the surface of the sporozoite, merozoite or gametocyte stages of the parasite lifecycle [305]. These include the pre-erythrocytic vaccine candidates RTS,S (the only malaria vaccine to pass phase III trials) [401, 402] and ME-TRAP [210, 403, 404] that are based on the circumsporozoite protein (CSP) and a string of epitopes fused to thrombospondin-related adhesion protein (ME-TRAP) respectively. These proteins localise to the surface of the sporozoite and play a role in the gliding motility and hepatocyte infectivity of sporozoites [234, 405, 406]. Similarly, several blood-stage vaccine candidates are based on proteins with known merozoite surface localisation such as the FMP1/AS02 vaccine (based on MSP-1<sub>42</sub>, the C-terminal fragment of MSP-1), combination B vaccine (based on a combination of MSP-1, -2 and RESA) and the GMZ2 vaccine formulations (based on a combination of MSP-3 and GLURP proteins) [305, 407, 408]. A transmission blocking vaccines based on *Pfs25*, a protein on the surface of gametocytes, is also under evaluation in phase I studies [229].

While several surface exposed antigens are already under consideration as malaria vaccine candidates, only very few of the potential total number of candidates have been identified and investigated. The *P. falciparum* genome from the 3D7 strain contains 5398 protein-coding genes [206], excluding pseudogenes. Of these genes, 1088 (20%) and 1754 (32%) are predicted to encode proteins with a signal peptide or at least one transmembrane domain respectively (<http://plasmodb.org/plasmo/>), and therefore could be potentially surface-associated proteins. As discussed in section 2.5.1, I mined data from PlasmoDB to identify merozoite antigens that could be potential vaccine candidates either by being secreted or located on the surface of *P. falciparum* merozoites. I limited the list to parasite proteins whose microarray

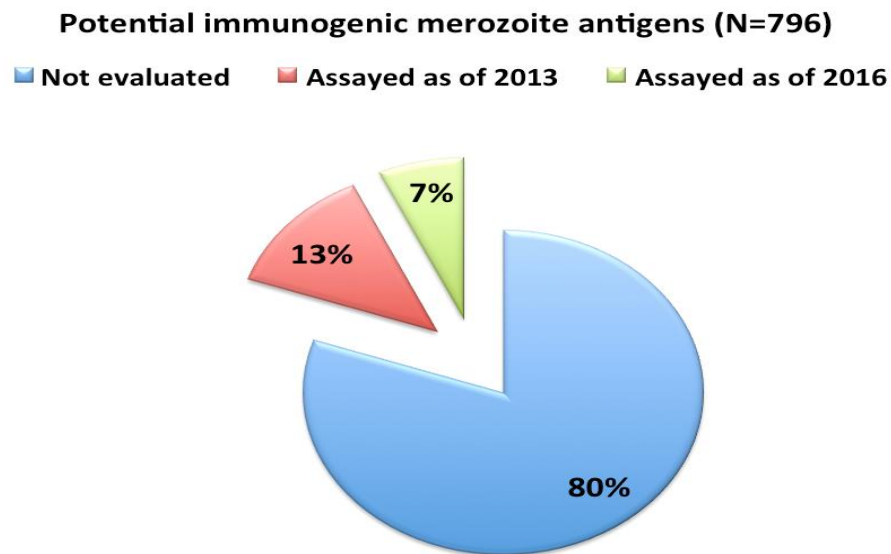
expression profiles indicated that maximum mRNA transcription occurs in the late-trophozoite, early to late schizogony or merozoite stage of the erythrocytic life cycle [259, 260]. This profile is similar to that of known merozoite surface proteins such as the GPI-anchored MSP-1, -2, *Pf92*, *Pf113* and *Pf12*. In addition, I limited the list of genes to those with a predicted signal peptide and/or predicted transmembrane domains that would indicate the protein could potentially be: i) secreted ii) peripherally associated to the merozoite surface or iii) an integral protein found on the merozoite surface. I found a total of 796 proteins with 550 (69.1%) containing a predicted signal peptide and 571 (71.7%) having at least one predicted transmembrane domain. These data indicate that there are potentially many novel merozoite surface proteins yet to be identified, that could be the target of protective antibodies. Furthermore, only 157 (20%) of these targets had been evaluated for antibody reactivity using sera from naturally exposed individuals (Figure 3.1). However, the evaluation of each of the remaining 639 antigens for their ability to elicit protective antibody responses would be a daunting task within the time constraints of a PhD.

Membrane proteomics represents a potential approach to down-select the list of bioinformatically predicted surface proteins. These approaches have been employed to attempt to comprehensively identify surface proteins on *P. falciparum* erythrocytes [299] and on the surface of *P. falciparum* sporozoites, resident in mosquito salivary glands [300]. Biochemical purification and proteomics has also been used to identify novel antigens that are membrane or GPI-anchored to the surface of merozoites [294-296]. These approaches led to the identification of the novel GPI-anchored proteins such as *Pf12*, *Pf92* and *Pf113*, as well as the *cys6* proteins *Pf38* and *Pf41* that are peripherally associated to the merozoite surface. Some of these targets were subsequently shown to be immunogenic using sera from individuals naturally infected with *P. falciparum* malaria [146, 147] and antibodies to these targets were associated with protection from clinical malaria individually in Kenyan and Papua new guinea children. Membrane proteomics therefore represents a validated mechanism to identify malaria vaccine antigens. In this section of my

PhD work, I employed a combination of proteomic approaches to describe the merozoite surface proteome and prioritize surface-exposed proteins for further characterization in the context of protective immunity against *P. falciparum* malaria.

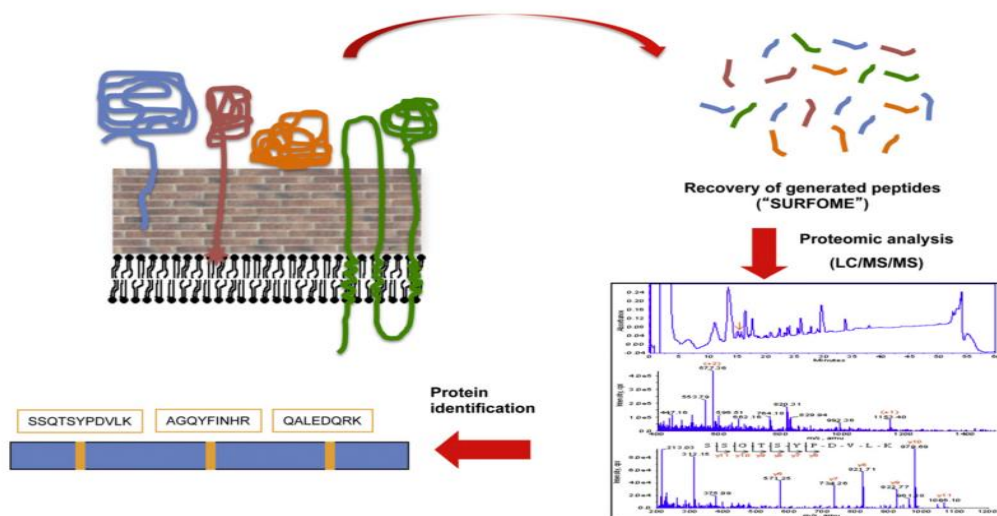
### **3.1. Rationale**

In this chapter, I sought to describe the merozoite surface proteome using two complementary techniques namely: i) a surface shaving and ii) surface biotinylation coupled to affinity purification. Mass spectrometry was used in combination with both approaches to identify targets. These two approaches have been successfully used to identify potential vaccine candidates, virulence factors and binding ligands in other infectious agents particularly bacterial organisms such as *Enterococcus faecium* [409], *Staphylococcus aureus* [410] and *Streptococcus* group A bacteria [400]. More recently, these approaches have been used to identify potential drug and vaccine targets in fungal organisms such as *Candida parapsilosis* and *Candida tropicalis* [411] and the parasites *Trypanosoma cruzi* [412] and *Schistosoma mansoni* [413]. In the context of *P. falciparum* malaria, the surface-shaving method has not been previously employed to elucidate surface proteins however; the surface biotinylation has been used to identify proteins on infected erythrocytes [299] and recently on sporozoites from mosquito salivary glands [300, 301].



**Figure 3.1: Potential *P. falciparum* merozoite targets predicted to be surface-embedded or exposed to antibodies.** A small proportion 157 (19.7%) has been evaluated for immunogenicity as of September 2016.

The surface shaving approach, also referred to as surfomics [414], is a rapid and direct method of identifying surface proteins in intact cells using proteolytic enzymes [415]. Target cells are incubated briefly with a proteolytic enzyme and the released proteins and peptides are then identified by mass-spectrometry as shown in Figure 3.2. Trypsin is the proteolytic enzyme chosen for the majority of surface shaving experiments because it specifically cleaves the carboxyl terminal of a lysine or arginine residues (positively charged amino acids) that are under-represented in hydrophobic domains. As a result, it is not thought to cleave peptides embedded within the hydrophobic lipid bilayer of membranes [392, 414].

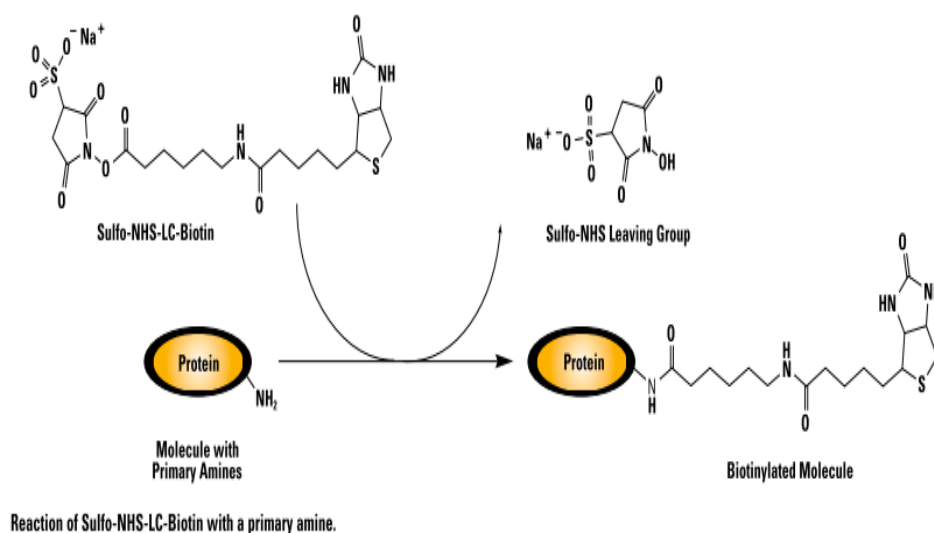


**Figure 3.2: Cell surface trypsinization strategy.** Workflow showing the general strategy employed when identifying surface-exposed proteins using the surface shaving approach coupled to mass-spectrometry identification of released targets [414].

The surface biotinylation approach relies on the use of a commercially available cell-membrane impermeable biotinylation reagent called EZ-link Sulfo-NHS-LC-Biotin [416]. This water-soluble reagent reacts efficiently with  $\alpha$ -amino groups present on the N-termini of all proteins and the  $\epsilon$ -amine group on lysine residues resulting in a biotinylated protein as shown in Figure 3.3. The presence of the sulfonate group ( $-\text{SO}_3$ ) on the reagent serves two important roles; 1) it allows the reagent to be dissolved in water-based buffers that are common in biological experiments and 2) the negative charge does not allow the reagent to penetrate the plasma membrane, thereby only labeling proteins on the surface. Once biotinylated, labeled proteins can be purified by affinity purification using avidin-based reagents such as agarose beads conjugated to streptavidin. This affinity purification exploits the naturally occurring non-covalent interaction between avidin and biotin. Affinity purified biotinylated proteins can then be identified by mass-spectrometry [416].

These two approaches have the potential to down-select the number of potential surface proteins identified through bioinformatics to those that can be confidently expected to be surface-exposed and thus warrant further evaluation. An additional strength of these combined techniques would be the ability to identify atypical

surface proteins that lack the regular signals for extracellular localization. These are the so-called “moonlighting” proteins [417] that may have been overlooked if we relied solely on bioinformatic or predictive algorithms to identify potential surface-proteins.



**Figure 3.3: Biotinylation of surface proteins: Addition of biotin to primary amine groups on the N-terminal of proteins [416].**



### 3.2. Objective

The overall objective was to identify the proteins located on the surface of *P. falciparum* merozoites using a combination of proteomic approaches

### 3.3. Specific objectives

- To identify proteins on the surface of *P. falciparum* merozoites using a surface shaving technique coupled to mass-spectrometry.
- To identify proteins on the surface of *P. falciparum* merozoites using surface biotinylation, affinity purification and mass spectrometry.

### 3.4. Laboratory methods

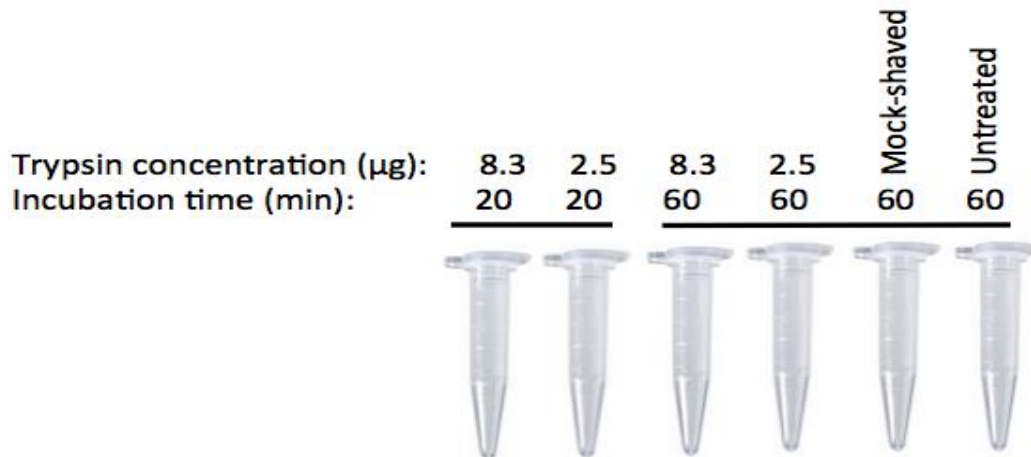
#### 3.4.0. *P. falciparum* merozoite purification

I obtained merozoites from *P. falciparum* 3D7 strain using the same protocol I developed in section 2.4.1 with the modification that once merozoites were pelleted, they were washed thrice in ice-cold phenol-red free RPMI media to remove the excess bovine serum albumin used as a supplement in culture and resuspended in phenol-red free RPMI media.

#### 3.4.1. Merozoite surface trypsinization

To identify the optimal conditions for cell surface trypsinization, I modified two key parameters that influence enzyme activity namely; 1) trypsin concentration and 2) incubation time for trypsin digestion. Cell surface trypsinization of merozoites was done as follows. Merozoites were washed twice in ice-cold phenol-free RPMI media and split into 6 equal fractions each containing 25µl of merozoites. As shown in Figure 3.4, in the first two fractions, 8.3µg and 2.5µg of trypsin was added and incubated at room temperature with rotation for 20 minutes. In the second set of two fractions, a similar trypsin concentration was added but with the incubation time increased to 60 minutes. The last two fractions served as controls in which one was

an untreated merozoite pellet (no trypsin added, no rotation, incubated at room temperature), while the second was a “mock-shaved” fraction, where no trypsin was added, but the merozoites were incubated at room temperature for 60 minutes with the same gentle rotation as the trypsin shaved fractions. After incubation periods were complete, treated merozoites were then pelleted by centrifugation at 5000 rpm for 4 minutes and supernatant (containing surface-shaved proteins and proteins that have either been shed or released due to cell lysis or damage) centrifuged further for 20 minutes at 20238xg. The two concentrations were selected following observations made in earlier experiments where 10-100µg of trypsin were used to treat merozoites resulting in a large degree of total protein cleavage as evaluated by Coomassie staining of the merozoite pellet material (data not shown).



**Figure 3.4: Optimisation of the merozoite cell surface trypsinization experiment.** Schema of the optimisation protocol tested to optimise the trypsin concentration and the incubation time for *P. falciparum* merozoite cell surface trypsinization.

To monitor the specificity of the surface-shaving protocol, shaved, untreated and mock-shaved pellet fractions were assessed by Western blot as described in section 2.4.2. I used the assay to detect the following proteins in the treated merozoites: i) detection of the endoplasmic reticulum resident protein, ERD2, which was used as a loading and negative control as I expected that it would not be cleaved following treatment, as it is intracellular and ii) merozoite surface protein, MSP3, as I

expected it would be cleaved in the trypsinized merozoites and only present in the untreated and mock-shaved fractions. Briefly, the Western blot assay was performed as follows; trypsinized and control treated merozoite pellets were resolved on a 4-12% gradient SDS gel (Invitrogen) and transferred onto a nitrocellulose membrane (Amersham Biosciences) overnight at 4°C at 30V. Nitrocellulose membranes were then blocked for 1 hour at room temperature with 5% non-fat milk/0.1% Tween 20 followed by incubation with anti-MSP3 or anti-ERD2 for 3 hours at room temperature at a 1:1000 dilution. Membranes were then washed three times in PBS/0.1% Tween 20 followed by incubation with anti-rabbit-HRP secondary antibody at a 1:10000 dilution (Sigma Aldrich) for 2 hours at room temperature. Membranes were washed three times in PBS/0.1% Tween 20 followed by a final wash in PBS. Detection of parasite protein was observed by developing the membrane using the ECL substrate (Amersham Biosciences) according to manufacturer's instructions' and visualized using autoradiography.

Once the trypsinization protocol was optimised, two independent biological replicates were generated for mass-spectrometry analysis as follows. Merozoites were resuspended in phenol-free RPMI media and split into 2 equal fractions. The sample to be trypsin-shaved was supplemented with 1µg of trypsin (proteomics grade, Sigma Aldrich), while the mock-shaved fraction was supplemented with 1µl of RPMI buffer; both fractions were then incubated for 20 minutes with rotation at room temperature. Treated merozoites were then pelleted by centrifugation at 5000 rpm for 4 minutes and the supernatant (containing surface-shaved proteins) centrifuged further for 20 minutes at 20238xg. Collected supernatants were stored in low protein binding Eppendorf tubes and 1µg of trypsin was added to the supernatant collected from the mock-shaved fraction to generate peptides. Thereafter, 2mM of the reducing agent TCEP was added to each supernatant fraction and stored at -20°C before identification by mass-spectrometry. The remaining pellets were immediately resuspended in sample buffer and heated at 95°C for characterization by Western blotting with anti-MSP3 and anti-ERD2.

### **3.4.2. Merozoite surface biotinylation and affinity purification**

A second approach was employed to identify merozoite surface proteins, which relied on the biotinylation of surface proteins using the cell membrane impermeable reagent EZ-link Sulfo-NHS-LC-Biotin (Thermo-Scientific). Several protocols were attempted to identify the best-suited protocol for extraction of biotinylated surface proteins for analysis by mass-spectrometry. In the first protocol, purified *P. falciparum* merozoites were washed once with ice-cold 1XPBS and incubated with 2mM EZ-link Sulfo-NHS-LC-Biotin for 30 minutes at room temperature. The biotinylation reaction was stopped and excess biotin quenched by adding 100mM glycine. Biotinylated merozoites were washed thrice in ice-cold 100mM glycine before extraction of proteins with different extraction buffers to solubilize biotinylated membrane proteins. These buffers included a 4% SDS lysis buffer, 8M Urea buffer, RIPA buffer and NP-40 buffer. Following extraction, proteins in the supernatant were incubated with streptavidin agarose beads (Pierce) (1ml of packed agarose resin for 3mg of biotinylated protein) for 1 hour at 4°C to allow non-covalent binding to biotinylated proteins. Prior to binding, agarose beads are washed thrice in ice-cold PBS to remove the storage buffer that contains ethanol. The resin was packed into a column and washed five times with ice-cold 1XPBS and biotinylated proteins eluted using various elution buffers for the ability to disrupt the strong non-covalent bond between biotinylated proteins and streptavidin. The elution buffers tested included sample buffer (lithium dodecyl sulphate, pH.8.4 containing 50mM dithiothreitol or 8M Guanidine HCL, pH 1.5.

In a second protocol, purified *P. falciparum* merozoites were similarly washed with ice-cold PBS, incubated with 2mM Biotin and quenched with 100mM glycine as has been previously described. To extract biotinylated proteins, merozoites were resuspended in distilled water containing protease inhibitors and lysed by 3 freeze-thaw steps using liquid nitrogen. After lysis, merozoite pellets were washed twice with protease inhibitors diluted in water and resuspended in 2% Triton X-100 then vortexed and left on ice for 1 hour. The extracted proteins in the supernatant were incubated with washed streptavidin agarose beads overnight at 4°C for affinity

purification. Following binding, agarose beads were packed onto a column and washed thrice with 1% Triton X-100/PBS followed by 3 additional washes with 1% Triton X-100 in 1M NaCl. Biotinylated proteins were eluted by incubating with 5ml of 8M Guanidine HCL, pH 1.5 for 1 hour at room temperature and then neutralised with 5ml of 250mM Tris, pH. 7.4. Proteins were then concentrated using a 3K MWCo concentrator (Pierce, Thermo Scientific). To monitor the biotinylation experiments, the following fractions were collected i) the biotinylated merozoite proteins, ii) flowthrough following binding, iii) washes to release unbound proteins and iv) eluates containing biotinylated proteins. Western blotting as has been described in section 2.4.2 was used to test the fraction collected. Briefly, the fractions were resolved on a 4-12% gradient SDS gel and transferred onto nitrocellulose membrane and probed with either streptavidin conjugated to HRP, anti-MSP3 or anti-ERD2.

#### ***3.4.3. LC-MS/MS analysis, database search and in-silico analysis of protein sequences***

Peptides obtained following the surface-shaving experiments were analysed using an Ultimate 3000 Nano/Capillary LC System (Dionex) coupled to an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher) equipped with a nanospray source. Supernatant containing surface-shaved and mock-shaved trypsin digested peptides were desalted using the C18-stage tips and acidified to a final concentration of 1% trifluoroacetic acid. The samples were then loaded and desalted on a PepMap C18 trap column (0.1mm id x 20mm, 5µm, Dionex) for 20 minutes at a 10µl/min flow rate prior to loading on the separation column (PepMap 75µm id x 25cm column with a 5µm particle size, Dionex). The samples were separated over a 120-minute long-linear gradient changing from 100%ACN/0.1%v/v formic acid (FA) in water to 5-45% of buffer B (80%ACN/0.1%FA) before a final elution using 95% buffer B to release the hydrophobic peptides. The Orbitrap mass spectrometer was operated in the “top 15” data-dependant acquisition mode while the preview mode was enabled. The MS full scan was set at m/z of 380-1600 with a resolution at 30,000 at m/z 400 and the automatic gain control (AGC) set at  $1 \times 10^6$  with a maximum injection time of 200 msec. The 15 most abundant multiply-charged precursor ions,

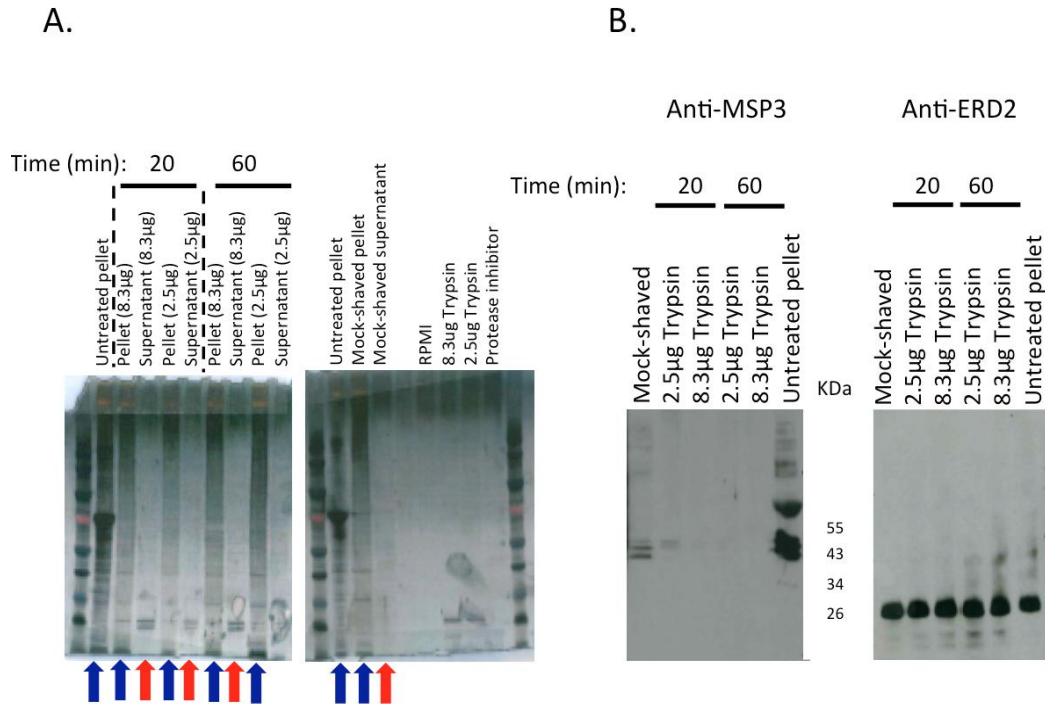
with a minimal signal above 3000 counts, were selected for CID fragmentation (MS/MS) in the LTQ Velos ion trap, which has the AGC set at 5000 and a maximum injection time at 100ms. The raw files were processed in Proteome Discoverer (V1.4) (Thermo Fisher) using Mascot (V2.5) (Matrix Science) as the protein database search engine with the following parameters: Enzymatic digestion was set to trypsin with a maximum of 2 missed cleavages sites; peptide mass tolerance search was set at 20 ppm; MS/MS fragment mass tolerance at 0.50 Da. Variable modifications for Acetyl (Protein N-term), Carbamidomethyl (C), Deamidated (NQ), and Oxidation (M). The protein databases were downloaded from Uniprot (October 2014) plus the common contaminating database. False Discovery Rate (FDR) setting was based on PEP at 0.01 in Percolator, and proteins with high confidence peptides were reported. Eluates from two biological replicates of the surface-biotinylation experiment were reduced to disrupt disulfide bonds using 20mM TCEP at 56°C for 1 hour. Thereafter, alkylation of cysteine to prevent reformation of disulfide bonds was performed by addition of 40mM iodoacetamide (IAA) for 45 minutes in the dark followed by addition of trypsin at a 1:40 E: S ratio at 37°C overnight. The peptides obtained were analysed as has been described above for the surface-shaving experiments.

The proteins identified by the database search were subjected to *in silico* analyses using publicly available algorithms for detecting a predicted signal peptide and transmembrane domains <http://www.cbs.dtu.dk/services/SignalP>, <http://www.cbs.dtu.dk/services/TMHMM/> respectively. The transcript levels of identified genes were derived from microarray transcriptome data available on PlasmoDB (<http://plasmodb.org/plasmo/>). Gene ontology cellular component enrichment analysis was performed using PlasmoDB where the frequency of occurrence of a particular cellular component term within the list of identified proteins is compared to the background frequency of occurrence in the total proteome.

### 3.5. Results

#### *3.5.1. Optimising the merozoite cell surface trypsinization protocol*

As a first step, I optimised the merozoite cell surface trypsinization protocol by varying the concentration of trypsin and the incubation time. As shown in Figure 3.5A (blue arrows), pellets obtained from short-term trypsin treatment contained proteins of varying sizes, which were not visible in the respective supernatant fractions (red arrows), suggesting that trypsin was not resulting in large scale lysis. I monitored the specificity of the trypsinization protocol by detecting the retention of the internal protein ERD2 and the loss of the peripherally associated merozoite surface protein, MSP3. As shown in Figure 3.5B, ERD2 was detected in all the pellets, including merozoites incubated with either 8.3 $\mu$ g or 2.5 $\mu$ g of trypsin, suggesting as expected, trypsin was not cleaving intracellular proteins. The MSP3 protein was detected in the merozoite pellet that was untreated and in the mock-shaved pellet fraction. Most pellet fractions to which trypsin had been added lacked MSP3, showing surface shaving had been successful. The exception was the 2.5 $\mu$ g of trypsin/20 minute's fraction, where the intensity of the MSP3 band was reduced relative to the mock-shaved fraction, indicating some loss of merozoite surface proteins, but not completely absent.



**Figure 3.5: Optimising the *P. falciparum* merozoite cell surface trypsinization protocol.** A) Silver-stained gel of the pellet and supernatants from the different treated merozoite fractions. Blue arrows show pellet material with proteins of varying sizes while red arrows are supernatant material in which fewer proteins were detected. B) Western blot results of the detection of MSP3 and ERD2 in pellets obtained from merozoites treated with trypsin, or mock-treated or left untreated.

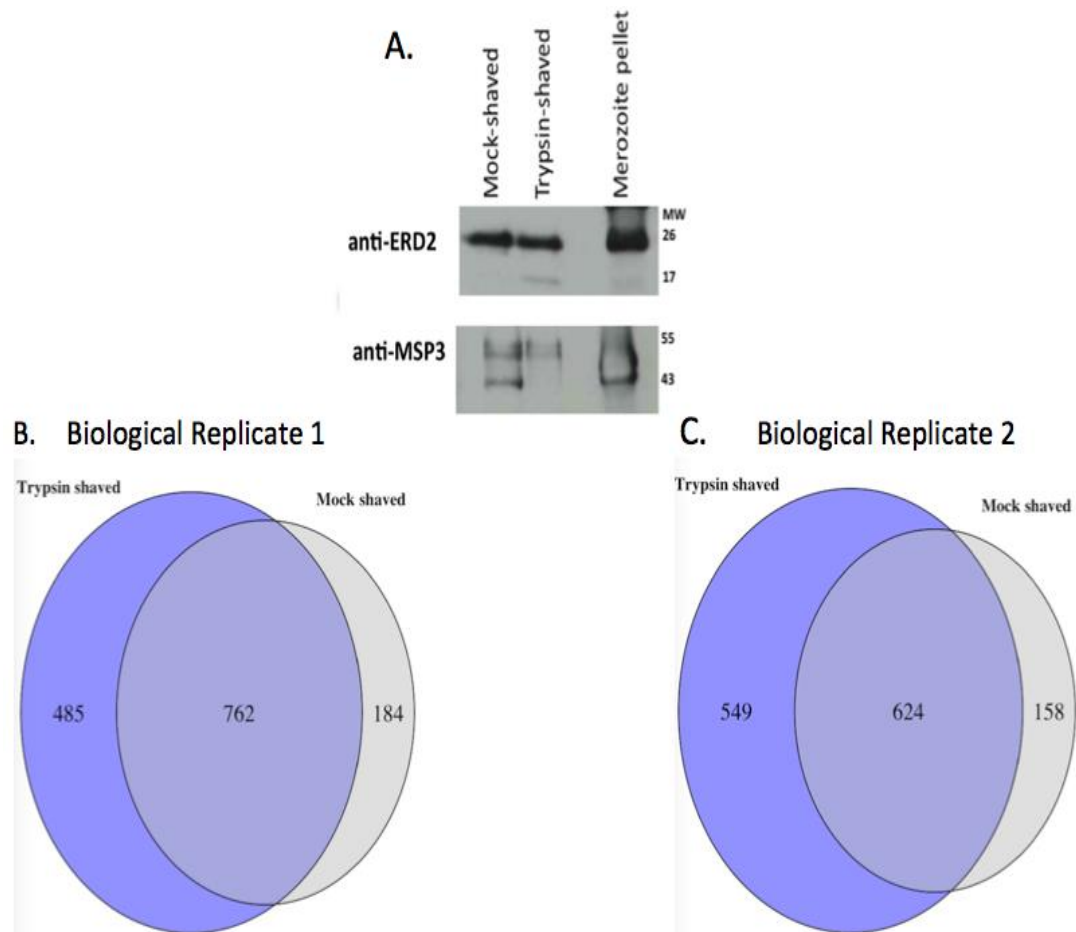
### 3.5.2. Putative merozoite surface proteins identified by cell surface trypsinization

To identify merozoite surface proteins, I performed two independent biological replicates of the merozoite trypsinization experiment and proteins released into the supernatant were identified by mass-spectrometry. Prior to protein identification, I confirmed the specificity of the trypsinization by detecting the presence of ERD2 in both fractions and the loss of MSP3 in the trypsin treated fraction as shown in Figure 3.6A. In one biological replicate, a total of 1857 proteins were identified from supernatant obtained from trypsin shaved and mock-shaved merozoites. I eliminated proteins that were not *P. falciparum* specific (N=33), often referred to as contaminants i.e. keratin, trypsin, proteins from *Bos Taurus* (bovine origin) or *Homo sapien* (human origin). I also eliminated proteins that were identified when



peptides were mapped onto a decoy database (database derived from reversing the *P. falciparum* database) (N=25) or proteins in which information on intensity (a measure of the quantity) was absent (N=353). Similarly, for the second biological replicate, I eliminated contaminant proteins (N=33), proteins that mapped onto the decoy database (N=11) and proteins in which information on intensity was absent (N=453). Therefore, the total number of *P. falciparum* specific proteins identified from supernatants obtained from the mock-shaved and trypsin treated merozoites in the two independent biological replicates was 1431 and 1331 respectively.

As shown in Figure 3.6B, 485 (33.9%) of proteins identified in the first experiment were uniquely identified in supernatant from trypsin-shaved merozoites while 184 (12.9%) were identified in the mock-shaved supernatant. The majority of proteins identified, 762 (53.2%) were detected in both fractions. In the second experiment, 549 (41.2%) and 158 (11.9%) were uniquely identified in the trypsin treated and mock shaved fractions respectively and 624 (46.9%) were identified in both fractions (Figure 3.6C). The list of proteins identified in the trypsin shaved, mock shaved and present in both fraction for the two biological replicates are found in Appendix 3.1 and 3.2 respectively. In both cases, relatively few proteins were specifically released upon trypsin treatment. These results were similar to findings from cell surface trypsinization experiments on the extracellular stage (trypomastigotes) of the blood-borne pathogen *Trypanosoma cruzi*. In this study, surface shaving identified 1245 targets of which 566 (46%) were found exclusively in the treated cells and 483 (39%) in both shaved and control trypomastigotes [412].



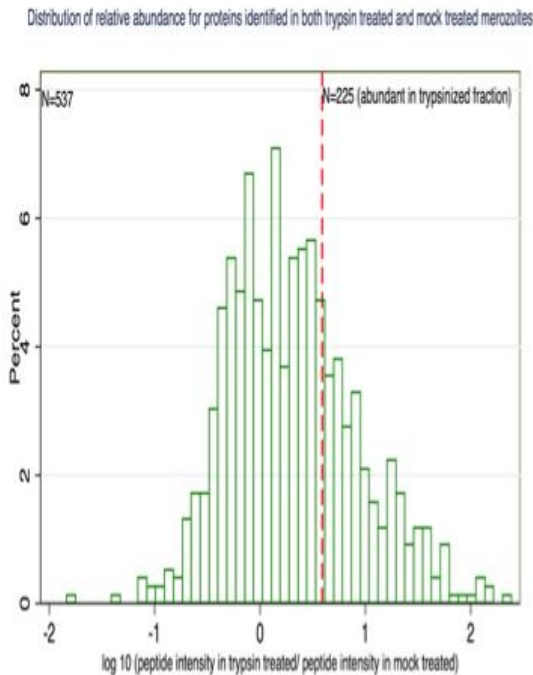
**Figure 3.6: Identification of putative merozoite surface proteins by cell surface trypsinization.** A) Western blot detection of retention of ERD2 and loss of MSP3 in trypsin treated merozoites. *P.falciparum* specific proteins identified in supernatant from trypsin treated and mock shaved merozoites in B) biological replicate 1 and C) biological replicate 2.

### 3.5.3. Defining potential *P. falciparum* merozoite surface proteins from surface shaving

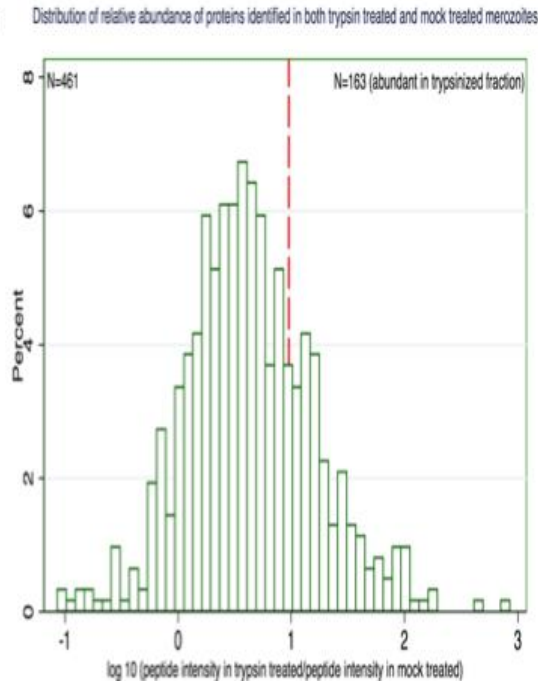
The two biological replicates were analysed independently and only merged after defining a putative surface protein list from each experiment as is described below in section 3.5.6. To obtain a list of putative surface merozoite proteins, several analyses were performed as follows. Proteins identified only in the trypsin-shaved fractions were all considered to be surface proteins. However, some proteins in the overlap between both the shaved and mocked fraction in the two replicates (Figure 3.6B and C; 762 and 624, respectively) could also be surface proteins. Several proteins present on the merozoites are shed prior to or at the point of invasion of

erythrocytes (e.g. MSP1 and AMA1) [408, 418], meaning that they would be found in both the shaved and mock-shaved samples. However, I hypothesized that trypsin treatment should accelerate the surface shedding process, and therefore cleaved surface proteins would be more abundant in the trypsin treated compared to mock shaved merozoites. I compared the relative abundance of the proteins in each of the fractions by calculating the intensity ratio (IR) as follows:  $RI = \text{intensity of protein in trypsin treated merozoites} / \text{intensity of protein in mock treated merozoites}$ . A RI greater than 1 would indicate that the protein identified was more abundant in the trypsin treated supernatant compared to the mock shaved merozoites. I then used a set of genes predicted to be intracellular to generate a cut-off ratio such that any protein with an RI value greater than the cut-off would be considered putative surface proteins.

### A. Biological replicate 1



### B. Biological replicate 2

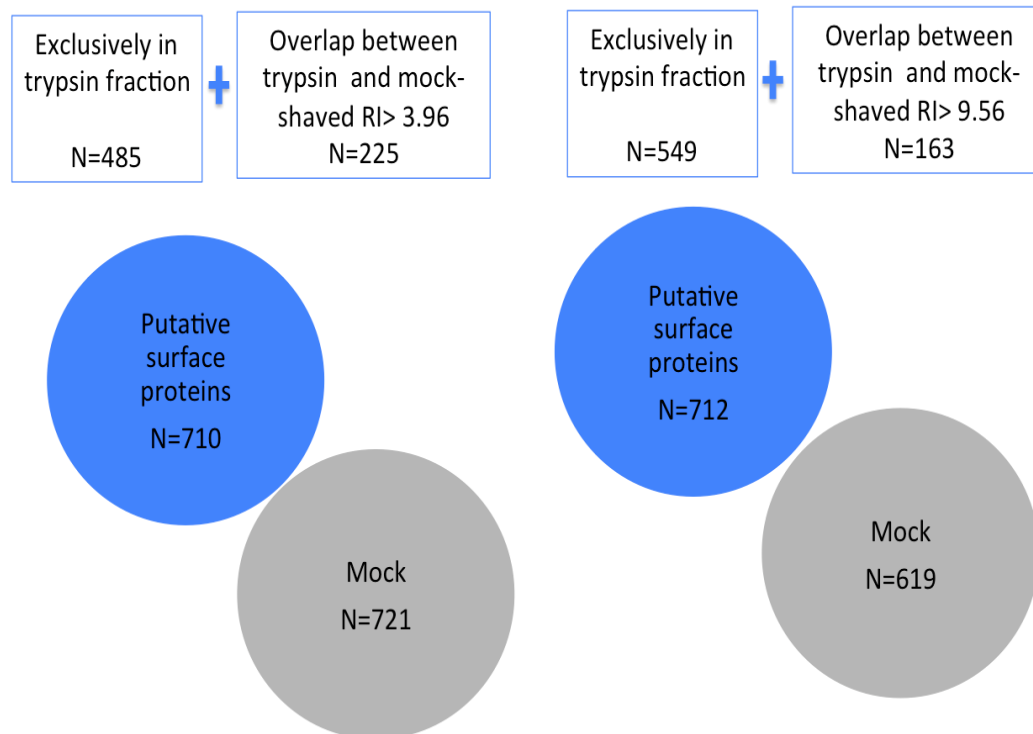


**Figure 3.7: Relative abundance of proteins identified in both the mock treated and trypsin treated merozoites in:** A) the first and B) the second biological replicate.

Proteins considered intracellular had been designated terms such as ‘Nuclear’, ‘Transcription’, ‘DNA’, ‘Helicase’, ‘Proteasome’, ‘Ribosomal’ and ‘Heat shock’. A total of 126 (16.5%) and 96 (15.4%) proteins were considered intracellular and resulted in a RI cut-off of 3.96 ( $\log_{10}(\text{RI})=0.59$ ) and 9.56 ( $\log_{10}(\text{RI})=0.98$ ). As shown in Figure 3.7A and B, a total of 225 and 163 proteins had an RI ratio above this cut-off point in the two biological replicates. This resulted in a set of 710 (225+485=710) and 712 (163+549) designated as putative surface proteins from the two biological replicates (Figure 3.8A and B, respectively). The remaining proteins, 721 and 619 targets were considered mock proteins. On searching the PlasmoDB database with the unique uniprot IDs, the 710 and 712 targets that were designated as putative surface proteins, mapped to 681 and 684 targets respectively. The list of proteins designated as putative surface proteins is available in Appendix 3.3.

#### A. Biological replicate 1

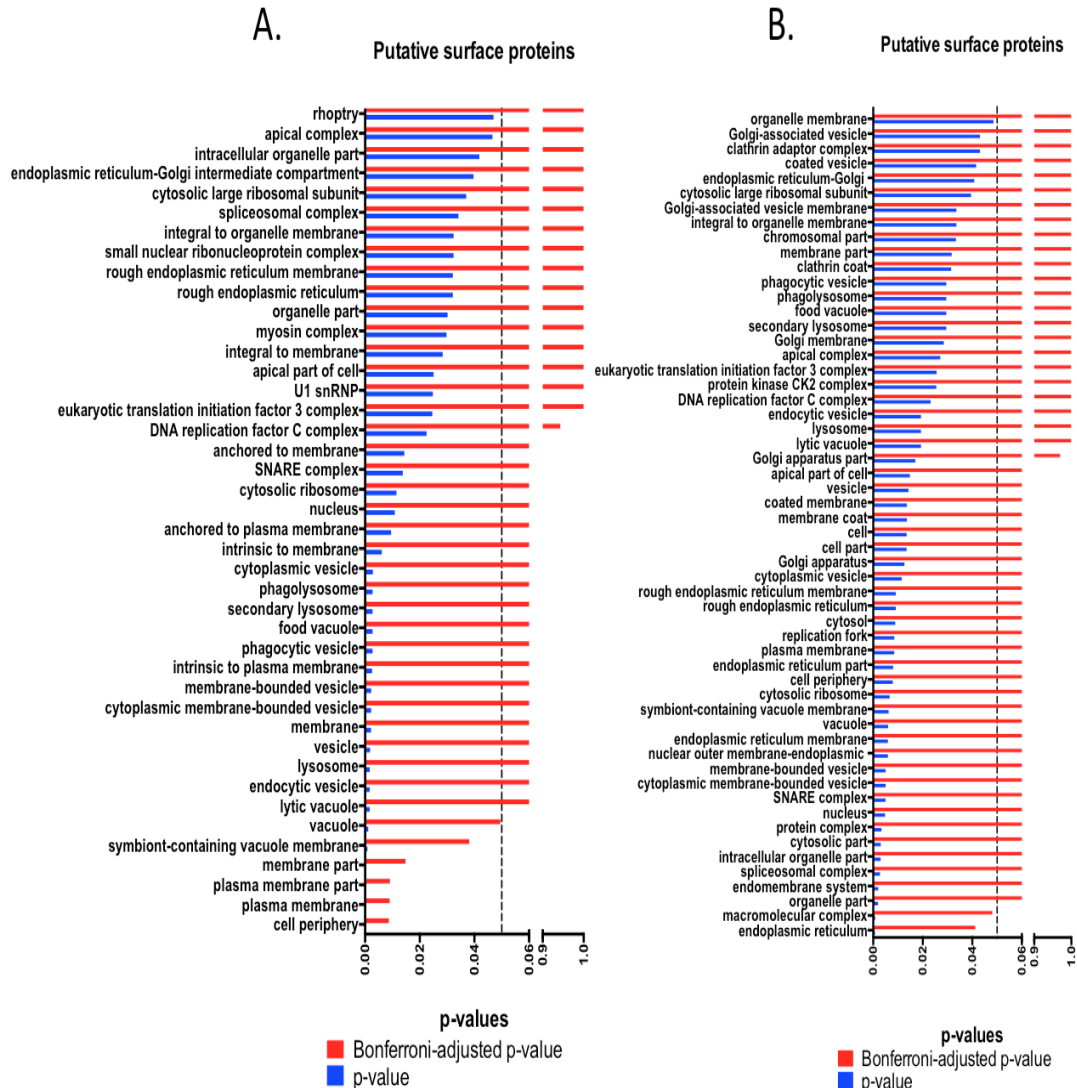
#### B. Biological replicate 2



**Figure 3.8: A summary of the number of targets re-classified at putative surface proteins or mock (intracellular) proteins.** Data from the first (A) and second (B) biological replicate of the surface-shaving experiments is presented.

#### ***3.5.4. Comparing the predicted gene ontology and level of polymorphism between putative surface and mock proteins***

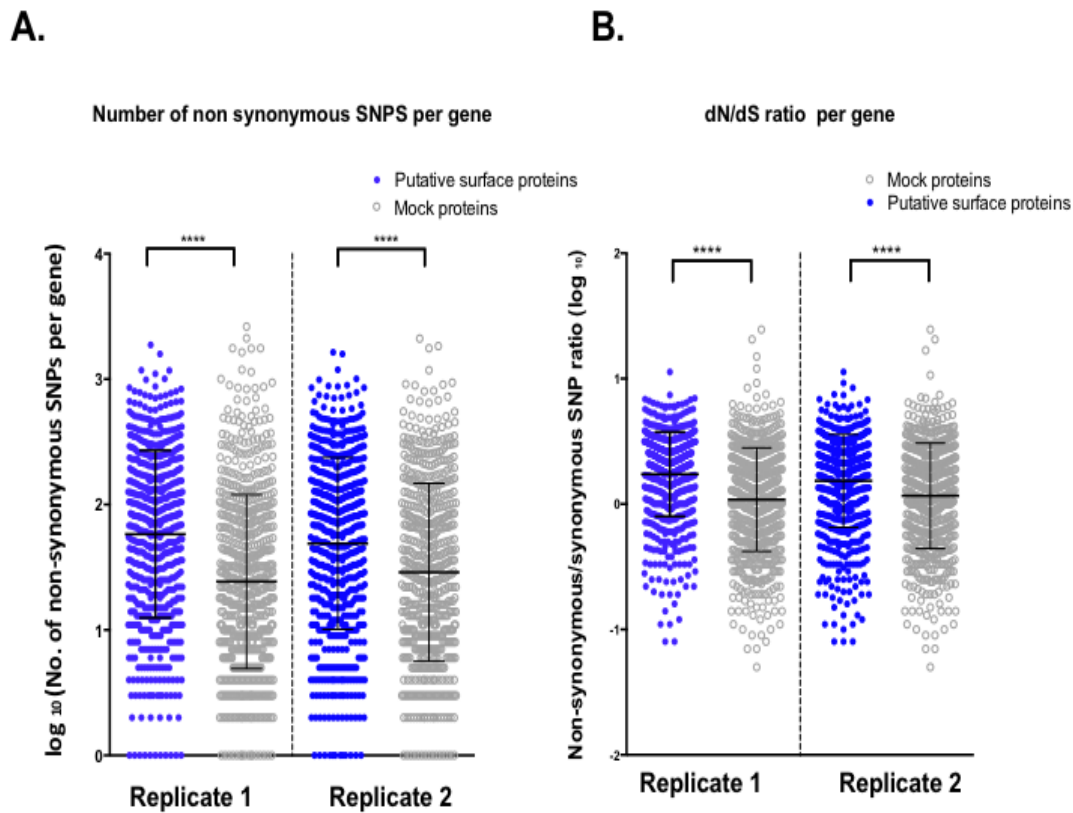
Having defined the set of proteins likely to be on the surface of the merozoite, I evaluated which cellular component terms were enriched within my list when compared to their occurrence in the entire 3D7 protein coding genes. This gene ontology cellular component enrichment analysis was performed using PlasmoDB, and it compares the frequency of occurrence of a particular cellular component term within my list of putative surface proteins compared to the background frequency of occurrence in the entire organisms set of genes. As shown in Figure 3.9 A and B, the two top cellular component terms that were enriched in the surface protein list from the first biological replicate was ‘cell periphery’ (p-value=0.0087) and ‘plasma membrane’ (p-value=0.0090), fitting in with the expectation of enriching for surface proteins. However, for the second biological replicate the two enriched component terms were ‘endoplasmic reticulum’ (p-value=0.0412) and ‘macromolecular complex” (p-value=0.0481), which were borderline significant. This may indicate that a higher degree of merozoite lysis occurred during the second replicate experiment.



**Figure 3.9: Gene ontology cellular component enrichment analysis in proteins designated surface proteins.** Data presented in the first A) and B) second biological replicate experiments. The enriched gene-ontology terms are arranged in order of descending significance. In A) significantly enriched terms are “cell periphery” and “plasma membrane” while in B) it was “macromolecular complex” and “endoplasmic reticulum” were borderline significant.

I hypothesized that proteins on the surface of the merozoite would be more polymorphic compared to mock proteins due to exposure to the host immune’s system. I therefore compared the number of non-synonymous SNPs (single nucleotide polymorphisms that result in an amino acid change) in genes classified as surface proteins and mock proteins. As shown in Figure 3.10A, putative surface proteins had a significantly higher number of non-synonymous SNPs compared to

mock proteins. The mean  $\log_{10}$  non-synonymous SNPs between surface and mock proteins were 1.764 versus 1.386 (p-value <0.0001: Unpaired *t* test) and 1.690 versus 1.460 (p-value<0.0001: Unpaired *t* test). Similarly, the mean  $\log_{10}$  ratio of non-synonymous /synonymous SNPs per gene (dN/dS ratio) was significantly higher in the surface proteins when compared to mock proteins. As shown in Figure 3.10B, the mean  $\log_{10}$  dN/dS ratio between surface and mock proteins was 0.2385 versus 0.0357 (p-value <0.0001: Unpaired *t* test) and 0.1849 versus 0.0653 (p-value <0.0001: Unpaired *t* test).



**Figure 3.10: Comparison of the number of non-synonymous SNPs per gene and the ratio of non-synonymous to synonymous SNPs in mock and putative surface proteins.** The number of non-synonymous SNPs (A) and the ratio of non-synonymous to synonymous SNPs (B) in genes encoding putative surface proteins were significantly higher than compared to mock proteins. The Mann-Whitney U test was used for comparisons. \*\*\*\*: p-value < 0.0001

### ***3.5.5. Comparing the protein features represented in putative surface and mock proteins***

To analyse further the characteristics of the proteins I identified (N=1651) in both biological replicates, I obtained data from PlasmoDB with information on the predicted gene ontology for each of the antigens as well the presence of a predicted signal peptide or transmembrane domains. In addition, I used data from a peer-reviewed publication [258] in which changes in gene expression following exposure to 20 parasite growth inhibitory compounds were analysed over the asexual erythrocytic cycle. A set of genes termed the “invadome” that may be important for erythrocyte invasion were proposed from this article. I then compared the occurrence of these features in the following ways: i) between different fractions within my dataset i.e. compared occurrence in putative surface versus mock proteins and ii) between the different fractions within my dataset and their occurrence within the entire genome.

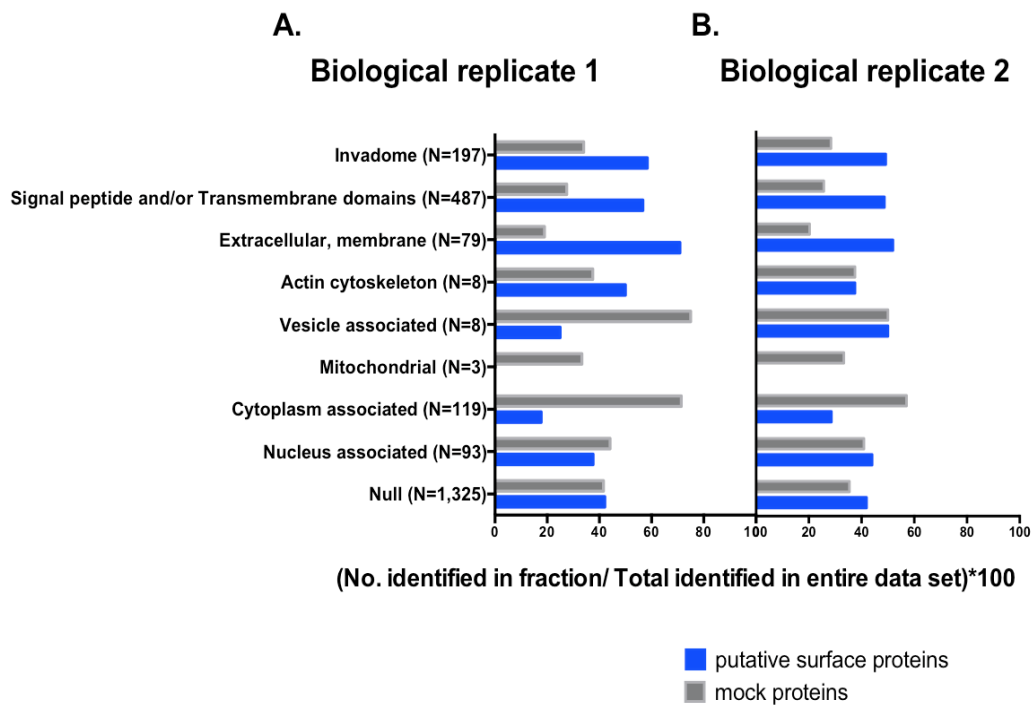
#### ***3.5.5.1. Comparison of features between putative surface and mock proteins***

As shown in Figure 3.11, features characteristic of surface-exposed proteins such as a predicted N-terminal signal peptide and/or transmembrane domains were generally enriched in proteins classified as putative surface proteins (the blue bars are longer than the grey bars). Similarly, the proportion of proteins predicted to be cytosolic, mitochondrial or vesicle located was larger in mock proteins (the grey bars are longer than the blue bars)

For example, as shown in Figure 3.11, 197 (12%) of the proteins I identified (in both mock and putative surface proteins) were proposed to be genes encoding proteins essential for merozoite invasion of erythrocytes [258]. Of these targets, 115 (58%) were identified in biological replicate 1 and 97 (49%) were identified in biological replicate 2, as putative surface proteins. These included the 6-cys proteins *Pf38* and *Pf41*, the GPI-anchored micronemal antigen (GAMA), glideosome-associated protein 40, 45 and 50 (GAP40, GAP45 and GAP50) and MSP-1, 9, 10 among others [258]. A small proportion of “invadome” genes were identified in



mock proteins in both biological replicates (67 (34%) and 56 (28%) respectively). These included proteins and enzymes that may play important roles prior to merozoite egress or invasion but are not surface-located. These include enzymes such as CDPK4, putative protein kinases, putative phosphatases, and subtilisin-like proteases 2 (SUB2) among others. Similarly, 487 (29%) of all the proteins identified (in both mock and putative surface proteins) were predicted to have a signal peptide and/or transmembrane domains which are features that suggest a protein localisation may be extracellular or on the surface of a cell. Of these targets, 276 (56%) were identified in biological replicate 1 and 237 (49%) were identified in biological replicate 2 as putative surface proteins. A small proportion of all identified proteins were predicted to be cytosolic 119 (7%), nucleus-associated 93 (6%) and mitochondrial 3 (0.2%). Interestingly, for the majority of targets 1325 (80%) I identified, there was no predicted sub-cellular localisation.



**Figure 3.11: Predicted cellular localisation and representation of protein features putative surface and mock proteins.** Data presented are proteins identified as putative surface proteins or the mock proteins in the first (A) and second (B) biological replicate. Features suggesting surface localisation such as signal peptide and/or transmembrane domains were represented to a higher proportion in putative surface proteins. Proteins predicted to be cytoplasmic located were enriched in mock proteins.

### 3.5.5.2. Comparison of features between putative surface/mock proteins and occurrence in the genome

I compared the occurrence of proteins with features suggesting surface localisation or roles in merozoite invasion of erythrocytes within the dataset obtained and in the genome. As shown in Table 3.1, proteins that were proposed to be members of the invadome were significantly enriched within the putative surface protein list when compared with occurrence within the entire genome. No differences were observed between the proportion of proteins with a signal peptide or transmembrane domains in the putative surface proteins when compared with the genome. However, the proportions of these proteins were significantly lower in mock proteins when compared to the genome. This validated the methods I used to differentiate between putative surface and mock proteins.

**Table 3.1: Table showing the proportion of proteins proposed to be members of the invadome or have a signal peptide and/or transmembrane domain found within the genome and datasets obtained from merozoite trypsinization**

Features	Proportion found within different datasets				
	Biological replicate 1				
	Proportion found within the genome# N=5305 (%)	Putative surface proteins N=710	P-value	Mock proteins N=721	P-value
<b>Member of the invadome</b>	418 (7.88%)	115 (16.19)	<b>0.0001</b>	67 (9.29%)	0.1917
<b>Presence of a SP and or TMDs</b>	1979 (37.3%)	276 (38.87)	0.4591	134 (18.59)	<b>0.0001</b>
	Biological replicate 2				
	Proportion found within the genome# N=5305 (%)	Putative surface proteins N=712	P-value	Mock proteins N=619	P-value
<b>Member of the invadome</b>	418 (7.88%)	97 (13.62)	<b>0.0001</b>	56 (9.05)	0.3100
<b>Presence of a SP and or TMDs</b>	1979 (37.3%)	237 (33.29)	0.0563	125 (20.19)	<b>0.0001</b>

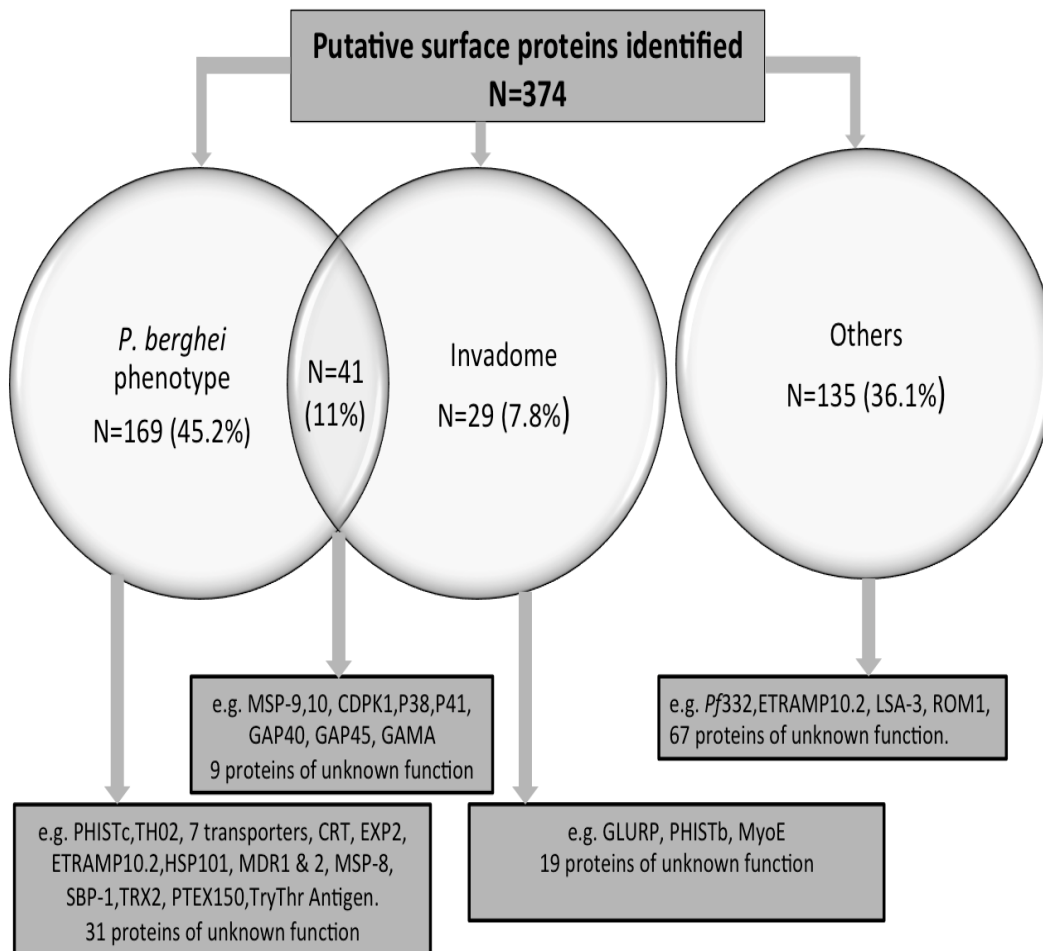
#- Protein coding genes on PlasmoDB excluding pseudogenes. SP: Signal peptide. TMDs: Transmembrane domains

### 3.5.6. Putative surface proteins identified in both biological replicates

To increase the likelihood of identifying true merozoite surface proteins, I narrowed down to the targets classified as putative surface proteins in both biological replicates, N=374. Of these targets, 157 (42%) were predicted to have an N-terminal signal peptide and/or at least one transmembrane domain. I then investigated the overlap between these putative surface proteins and the invadome [258] as well as with genes for which genetic manipulation had been attempted in their *P. berghei* orthologues, as of October 2016. Phenotypes obtained from these experiments have been deposited on the RMgMDB (<http://www.pberghei.eu/index.php>) and PlasmogEM (<http://plasmogem.sanger.ac.uk/phenotypes>). A summary of the overlap between proteins identified in my study, the invadome and *P. berghei* genetic modification database is shown as Figure 3.12. These represent additional information that could be considered when selecting potential vaccine candidates for evaluation from a list of putative merozoite surface proteins.

Among the putative surface proteins I identified in both biological replicates, 70 (19%) of them are considered members of the proposed invadome, as shown in Figure 3.12 and Table 3.2. These are genes whose transcriptional profiles were up or down-regulated during the asexual erythrocytic cycle following exposure to parasite growth inhibitory compounds. As shown in Table 3.3, the proportion of genes termed as invasion-related were significantly higher when compared to the occurrence within the genome: 18.7% versus 7.9% p-value <0.0001. Among these were known merozoite surface proteins under evaluation as potential blood-stage vaccine candidates such as the MSP-9 and 10, the 6-cys proteins P41 and P38 [294-296] and GAMA [419]. Additional proteins that have been shown to localise to the surface of free merozoites, and are considered essential for invasion that were identified as putative surface proteins in my study included, 1) PF3D7-0217500, also known as calcium-dependent protein kinase 1 (*Pf*CDPK1) that was shown to co-localise with MSP1 in indirect immuno-fluorescent assays [420], 2) PF3D7-0210700, also known as syntaxin (*Pf*Stx1), has been shown to co-localise with *Pf*MSP3 and 3) PF3D7-0206200 also known as the pantothenate transporter (PAT),

showed localisation to the surface of individual merozoites within schizonts [421]. None of these antigens have been evaluated in the context of naturally acquired immunity. In addition, 28 (40%) of proteins that are considered to be members of the invadome and that I identified as putative surface proteins in this study, were termed as “conserved plasmodium protein, unknown function” or “conserved plasmodium membrane protein, unknown function”. These represent potential targets of protective antibodies that have thus far not been evaluated.



**Figure 3.12: Publicly available data on the putative surface proteins identified.** Figure shows the overlap between putative surface proteins and a proposed set of invadome genes and *P. berghei* orthologues in whom genetic modification has been attempted. Examples of known and the number of understudied targets identified in each group in presented.

**Table 3.2: *P. falciparum* putative surface proteins identified in my study and are part of the invadome genes proposed by Hu *et al* [258].**

<i>Plasmodium falciparum</i> gene ID	Product description	No. of TMDs	Presence of SP
PF3D7_0204100	conserved Plasmodium protein, unknown function	0	1
PF3D7_0206200	pantothenate transporter (PAT)	11	0
PF3D7_0210600	conserved Plasmodium protein, unknown function	0	1
PF3D7_0210700	syntaxin, Qa-SNARE family (SYN17)	1	0
PF3D7_0217500	calcium-dependent protein kinase 1 (CDPK1)	0	0
PF3D7_0303200	HAD superfamily protein, putative	0	0
PF3D7_0308300	conserved Plasmodium protein, unknown function	0	0
PF3D7_0308700	conserved Plasmodium protein, unknown function	0	0
PF3D7_0321100	conserved Plasmodium protein, unknown function	0	0
PF3D7_0321400	protein kinase, putative	1	0
PF3D7_0403800	alpha/beta hydrolase, putative	0	0
PF3D7_0404900	6-cysteine protein (P41)	0	1
PF3D7_0407800	conserved Plasmodium protein, unknown function	0	0
PF3D7_0418600	regulator of chromosome condensation, putative	0	0
PF3D7_0420300	transcription factor with AP2 domain(s) (ApiAP2)	0	0
PF3D7_0423500	glideosome associated protein with multiple membrane spans 2 (GAPM2)	6	0
PF3D7_0423700	early transcribed membrane protein 4 (ETRAPM4)	2	1
PF3D7_0501600	roptry-associated protein 2 (RAP2)	0	1
PF3D7_0503600	myosin B (MyoB)	0	0
PF3D7_0506900	rhomboid protease ROM4 (ROM4)	6	0
PF3D7_0508000	6-cysteine protein (P38)	1	1
PF3D7_0515700	glideosome-associated protein 40, putative (GAP40)	10	0
PF3D7_0522600	inner membrane complex protein	8	1
PF3D7_0525800	inner membrane complex protein 1g, putative (IMC1g)	0	0
PF3D7_0528400	palmitoyltransferase (DHHC7)	4	0
PF3D7_0613900	myosin E, putative (myoE)	0	0
PF3D7_0618000	conserved Plasmodium membrane protein, unknown function	4	0
PF3D7_0620400	merozoite surface protein 10 (MSP10)	2	1
PF3D7_0722200	roptry-associated leucine zipper-like protein 1 (RALP1)	1	1
PF3D7_0730400	conserved Plasmodium protein, unknown function	0	0
PF3D7_0802600	adenyl cyclase beta (ACbeta)	0	0
PF3D7_0805300	conserved Plasmodium protein, unknown function	0	0
PF3D7_0813100	conserved Plasmodium protein, unknown function	0	0
PF3D7_0817700	roptry neck protein 5 (RON5)	0	0
PF3D7_0822900	conserved Plasmodium protein, unknown function	0	0
PF3D7_0828800	GPI-anchored micronemal antigen (GAMA)	2	1
PF3D7_0903600.1	conserved Plasmodium protein, unknown function	0	0
PF3D7_0908000	P1 nuclease, putative	2	0
PF3D7_0911100	conserved Plasmodium protein, unknown function	0	0
PF3D7_0913800	conserved Plasmodium protein, unknown function	0	0
PF3D7_0914400	conserved Plasmodium protein, unknown function	0	0
PF3D7_0918700	conserved Plasmodium protein, unknown function	0	0
PF3D7_0920500	ADP-ribosylation factor, putative	0	0
PF3D7_0930500	diacylglycerol kinase, putative (DGK1)	2	0
PF3D7_0935800	cytoadherence linked asexual protein 9 (CLAG9)	2	1
PF3D7_1014100	conserved Plasmodium protein, unknown function	0	1
PF3D7_1017500	conserved Plasmodium protein, unknown function	0	0
PF3D7_1023000	conserved Plasmodium membrane protein, unknown function	5	0
PF3D7_1031200	MORN repeat-containing protein 1 (MORN1)	0	0
PF3D7_1035300	glutamate-rich protein (GLURP)	0	1
PF3D7_1037500	dynammin-like protein (DYN2)	0	0
PF3D7_1113000	conserved Plasmodium protein, unknown function	0	0
PF3D7_1146600	CCAAT-box DNA binding protein subunit B (NFYB)	0	0
PF3D7_1222700	glideosome-associated protein 45 (GAP45)	0	0
PF3D7_1228600	merozoite surface protein 9 (MSP9)	0	1
PF3D7_1229300	conserved Plasmodium protein, unknown function	1	1
PF3D7_1250200	conserved Plasmodium membrane protein, unknown	9	1

<i>Plasmodium falciparum</i> gene ID	Product description	No. of TMDs	Presence of SP
PF3D7_1308000	conserved Plasmodium membrane protein, unknown function	4	0
PF3D7_1332200	conserved Plasmodium protein, unknown function	0	0
PF3D7_1343800	conserved Plasmodium protein, unknown function	2	0
PF3D7_1351700	inner membrane complex protein 1f, putative (IMC1f)	0	0
PF3D7_1410400	rhostry-associated protein 1 (RAP1)	0	1
PF3D7_1414500	atypical protein kinase, ABC-1 family, putative (ABCK2)	0	0
PF3D7_1452000	rhostry neck protein 2 (RON2)	1	1
PF3D7_1455300	conserved Plasmodium protein, unknown function	0	0
PF3D7_1460600	inner membrane complex sub-compartment protein 3, putative (ISP3)	0	0
PF3D7_1461400	conserved Plasmodium protein, unknown function	0	0
PF3D7_1463900	conserved Plasmodium membrane protein, unknown function	7	1
PF3D7_1467900	rab GTPase activator, putative	0	0
PF3D7_1476300	Plasmodium exported protein (PHISTb), unknown function	1	0

TMDs: Transmembrane domains. SP: Signal peptide

In a second analysis, I searched the rodent malaria genetic modification databases RMgmDB and PlasmoGEM to identify genes in which the *P. berghei* orthologue had either been tagged, disrupted/mutated or knocked down, or for which attempts to knock down were unsuccessful. I found a total of 210 of the putative merozoite surface proteins in these databases (67 (18%) and 184 (49%) in the RMgmDB and PlasmoGEM databases, respectively). Of these 210 targets, 41 of them have also been proposed to be essential for invasion as has been described above such as *PfPAT*, *CDPK1*, *P41*, *P38*, *MSP10* and *GAMA* among others.

#### 3.5.6.1. Overlap with targets with a phenotype reported in RMgmDB database

Of the 67 targets with a phenotype deposited on RMgmDB, three targets namely *PfPAT* [421], *ISP3* and *CDPK1* [420] have been shown to localise to the periphery of merozoites (Table 3.4), and therefore potentially accessible to circulating antibodies. In twenty-four (36%) of the 67 targets identified on RMgmDB database, efforts to knock down the gene have been unsuccessful despite several attempts as shown in Table 3.4. This includes 4 proteins namely PF3D7\_0305300, PF3D7\_1463900, PF3D7\_1455300 and PF3D\_1468100 that are described as “conserved *Plasmodium* (membrane) proteins, unknown function”. Eight targets had their *P. berghei* orthologues genetically disrupted, which resulted in a defect in merozoite membrane biogenesis formation or a reduced parasite growth rate over

the asexual blood stage. These targets included skeleton binding protein 1 (SBP1), Phosphatidylinositol 4-kinase (PI4K), Zn<sup>2+</sup> or Fe<sup>2+</sup> permease, Tyrosine kinase-like protein, putative (TKL4), *Plasmodium* exported protein (PHISTc), CCR4-associated factor 1 (CAF1), Heat shock protein 101 (HSP101), a putative transporter (PF3D7\_1129900) and Thioredoxin 2 (TRX2).

### 3.5.6.2. Overlap with targets with a phenotype reported in PlasmoGEM database

One hundred and eighty-four targets have had their *P. berghei* orthologue phenotypes reported on PlasmoGEM [336] (Appendix 3.4). These were targets identified from a high throughput reverse genetic screen using barcoded gene knockout vectors. Large pools of barcoded vectors were co-transfected simultaneously and a growth rate phenotype over the asexual stage of the life cycle assigned to genes. As a result, for a subset of the *P. berghei* genome, genes have been assigned as either essential, dispensable or result in a slow parasite growth rate over the erythrocytic stage [336]. As shown in Table 3.3, 104 targets are termed as essential and 38 as resulting in a reduced growth rate.

**Table 3.3: Table showing a comparison between the occurrences of proteins of specific characteristics within the putative surface protein data set and the genome.**

Feature	Proportion found within the genome# N=5305 (%)	Proportion found within the putative surface protein list N=374 (%)	P-value
Member of the invadome	418 (7.88)	70 (18.7)	<0.0001
<i>P. berghei</i> phenotype suggests the genes play essential functions over asexual stages%	1195 (46.4)	104 (27.8)	<0.0001
<i>P. berghei</i> phenotype suggests modification of the gene results in a reduced growth rate over asexual stages%	456 (17.7)	38 (10.2)	0.0003
<i>P. berghei</i> phenotype suggests the genes play dispensable roles over asexual stages%	911 (35.4)	41 (11.0)	<0.0001

#- Protein coding genes (excluding pseudogenes) on PlasmoDB. \*-N=5056 genes were analysed. %-N=2575 number of genes with a phenotype reported on PlasmoGEM.

**Table 3.4: *P. falciparum* putative surface proteins for which genetic manipulation has been attempted in *P. berghei* orthologues and phenotypes reported on RMgMDB.**

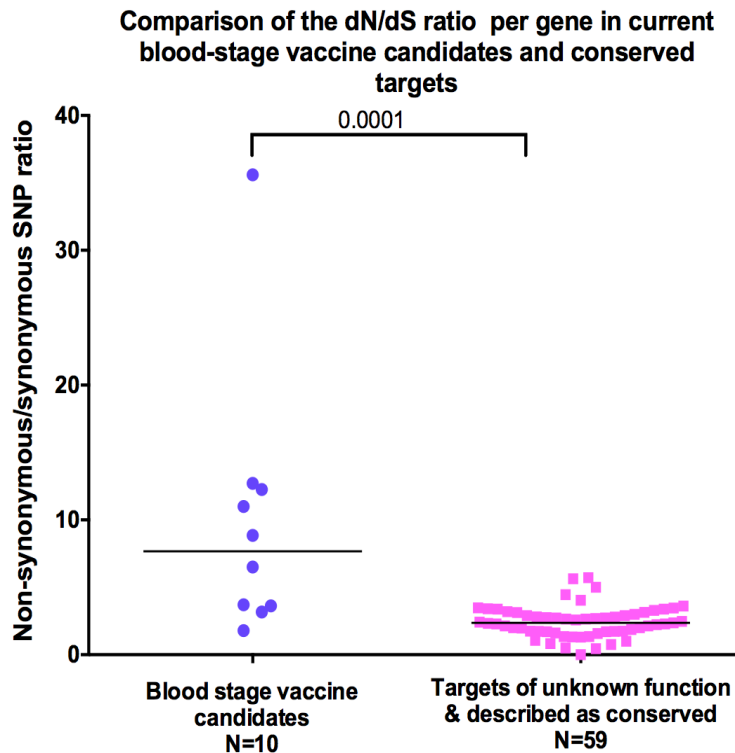
<i>Plasmodium falciparum</i> gene ID	Product description	Invadome	RMgmID	Rodent malaria gene ID	Modification attempted in rodent malaria parasites. *No publication.
Efforts to disrupt or knock-down the gene was not successful					
PF3D7_0305300	Conserved Plasmodium membrane protein, unknown function		RMgm-244	PBANKA_0403800	Two attempts to disrupt the gene were unsuccessful*
PF3D7_0321400	Protein kinase, putative	1	RMgm-575	PBANKA_1217100	Five attempts to disrupt the gene were unsuccessful[422].
PF3D7_0506900	Rhomboid protease ROM4 (ROM4)	1	RMgm-764	PBANKA_1106500	Three attempts to disrupt the gene were unsuccessful. M-cherry tagged ROM4 shows expression in schizonts and gametocytes[423].
PF3D7_0515700	Glideosome-associated protein 40, putative	1	RMgm-1402	PBANKA_1115300	Two attempts to disrupt the gene were unsuccessful[424].
PF3D7_0523000	Multidrug resistance protein (MDR1)		RMgm-1158	PBANKA_1237800	Three attempts to disrupt the gene were unsuccessful[425].
PF3D7_0628200	Protein kinase PK4 (PK4)		RMgm-737	PBANKA_1126900	Four attempts to disrupt gene were unsuccessful. Shown to be essential during the blood-stage of the life cycle[426].
PF3D7_0709000	Chloroquine resistance transporter (CRT)		RMgm-612	PBANKA_1219500	Four attempts to disrupt the gene were unsuccessful[427].
PF3D7_0904700	Bacterial histone-like protein (HU)		RMgm-266	PBANKA_0416700	Three attempts to disrupt the gene were unsuccessful[428].
PF3D7_0935800	Cytoadherence linked asexual protein 9 (CLAG9)	1	RMgm-248	PBANKA_0836300	Five attempts to disrupt the gene were unsuccessful*.
PF3D7_1007700	Transcription factor with AP2 domain(s) (ApiAP2)		RMgm-1130	PBANKA_1205900	Attempts to disrupt the gene were unsuccessful*. Number of attempts have not been reported
PF3D7_1037500	Dynamin-like protein (DYN2)	1	RMgm-765	PBANKA_0520400	Three attempts to disrupt the gene were unsuccessful.
PF3D7_1309200	Protein phosphatase 2c-like protein, putative		RMgm-1080	PBANKA_1407700	Five attempts to disrupt the gene were unsuccessful. GFP-tagged gene showed expression in asexual stages, gametocytes and oocysts[351].
PF3D7_1316600	Choline-phosphate cytidylyltransferase (CCT)		RMgm-339	PBANKA_1415100	Two attempts to disrupt the gene were unsuccessful. GFP-tagged CCT showed expression in the cytoplasm of blood-stages[343].
PF3D7_1320000	Rhoptry protein 2, putative (PRP2)		RMgm-215	PBANKA_1418300	Three attempts to disrupt the gene have been unsuccessful. GFP-tagged mature merozoites within schizonts showed two-dot staining characteristic of rhoptry-organelle localisation[345].
PF3D7_1323500	Plasmepsin V (PMV)		RMgm-881	PBANKA_1338700	Three attempts to disrupt the gene were unsuccessful*.
PF3D7_1340700	Ras-related protein Rab-11B (RAB11b)		RMgm-823	PBANKA_1354100	Two attempts to disrupt the gene were unsuccessful*.
PF3D7_1410400	Rhoptry-associated protein 1 (RAP1)	1	RMgm-1134	PBANKA_1032100	Attempts to disrupt the gene were unsuccessful*. Number of attempts have not been reported
PF3D7_1436300	Translocon component PTEX150 (PTEX150)		RMgm-1331	PBANKA_1008500	Three attempts to disrupt the gene were unsuccessful. HA-tagged PTEX150 showed localization to the parasitophorous vacuolar membrane (PVM)[429].
PF3D7_1450000	Serine/threonine protein kinase, putative		RMgm-541	PBANKA_1313700	Four attempts to disrupt the gene have been unsuccessful[422].
PF3D7_1452000	Rhoptry neck protein 2 (RON2)	1	RMgm-213	PBANKA_1315700	Three attempts to disrupt the gene were unsuccessful. mCherry tagged parasites showed apical localisation on merozoites[345].
PF3D7_1455300	Conserved Plasmodium protein, unknown function	1	RMgm-509	PBANKA_1319000	Two attempts to disrupt the gene were unsuccessful*.
PF3D7_1463900	Conserved Plasmodium membrane protein, unknown function	1	RMgm-190	PBANKA_1327100	Three attempts to disrupt the gene were unsuccessful.
PF3D7_1464600	Phosphatase, putative		RMgm-1364	PBANKA_1328000	Two attempts to disrupt the gene in the blood-stages have been unsuccessful[430].
PF3D7_1468100	Conserved Plasmodium protein, unknown		RMgm-510	PBANKA_1331400	Two attempts to disrupt the gene were unsuccessful*



Efforts to disrupt or knock-down the gene was successful					
<i>Plasmodium falciparum</i> gene ID	Product description	Invadome	RMgmid	Rodent malaria gene ID	Modification attempted in rodent malaria parasites
PF3D7_0206200	Pantothenate transporter (PAT)	1	RMgm-1098 RMgm-1372	PY17X_0304500 PBANKA_0303900	Gene disruption was successful resulting in no mature oocysts or sporozoite [424, 431]. <i>P. berghei</i> male and female gametes fail to egress from host erythrocytes after induction of gametogenesis[432].
PF3D7_0217500	Calcium-dependent protein kinase 1 (CDPK1)	1	RMgm-772	PBANKA_0314200	<i>P. berghei</i> GFP tagged CDPK1 shows expression in schizonts, gametocytes, ookinetes, oocysts and sporozoites[352]
PF3D7_0312500	Transporter, putative		RMgm-1383	PBANKA_0410500	Gene disruption showed reduced <i>P. berghei</i> sporozoite numbers <i>in vivo</i> [424].
PF3D7_0317100	6-cysteine protein (B9)		RMgm-929	PBANKA_0808100	Gene disruption resulted in arrested development of sporozoites within hepatocytes[433].
PF3D7_0320800	ATP-dependent RNA helicase DDX6 (DOZI)		RMgm-1583	PBANKA_1217700	Gene disruption showed no significant difference in growth rate with wild type parasites
PF3D7_0404900	6-cysteine protein (P41)	1	RMgm-480	PBANKA_1002600	Gene disruption showed no difference in growth rate compared to wild-type parasites[434].
PF3D7_0501300	Skeleton-binding protein 1 (SBP1)		RMgm-1452	PBANKA_1101300	C57B/6 mice infected with <i>PbΔsbp1</i> parasites did not develop cerebral malaria compared to those infected with wild type parasites. Reduced sequestration of schizonts in <i>PbΔsbp1</i> compared to wild-type[435].
PF3D7_0501600	Rhoptry-associated protein 2 (RAP2)	1	RMgm-209	PBANKA_1101400	GFP tagged parasites showed two-dot staining in merozoites within schizonts and in oocyst and salivary gland sporozoites[345].
PF3D7_0502400	Ring-stage membrane protein 1, merozoite surface protein 8 (MSP8)		RMgm-225	PBANKA_1102200	<i>P. berghei</i> parasites expressing truncated MSP8 showed no difference in growth rate when compared to wild-type parasites[346].
PF3D7_0503600	Myosin B (MyoB)	1	RMgm-1230	PBANKA_1103300	GFP tagged MyoB showed expression on the extreme apical end of merozoites within schizonts[436].
PF3D7_0508000	6-cysteine protein (P38)	1	RMgm-354	PBANKA_1107600	<i>PbΔp38</i> showed no differences from wild type across the complete life cycle[437].
PF3D7_0509800	Phosphatidylinositol 4-kinase (PI4K)		RMgm-969	PBANKA_1109400	Mutated gene showed that inhibition affects merozoite formation interfering with membrane biogenesis around developing merozoites[438].
PF3D7_0528400	Palmitoyltransferase (DHHC7)	1	RMgm-901	PBANKA_1243000	Gene disruption successful, however the growth rate comparison with wild type has not been determined[439].
PF3D7_0609100	Zn2+ or Fe2+ permease		RMgm-1143	PBANKA_0107700	Gene disruption was successful resulting in a reduced parasite multiplication rate over the asexual blood stage when compared to wild type parasites. Reduced numbers of female gametocytes, no male gametocytes, no ookinetes, oocyst or sporozoites develop within mosquitoes[424].
PF3D7_0614300	Organic anion transporter		RMgm-1395	PBANKA_0112500	Gene disruption successful and no differences observed across the asexual blood stage when compared to wild-type parasites[424].
PF3D7_0620400	Merozoite surface protein 10 (MSP10)	1	RMgm-416	PBANKA_1119600	No phenotype presented[434].
PF3D7_0623800	Tyrosine kinase-like protein, putative (TKL4)		RMgm-533	PBANKA_1122700	Gene deletion was successful resulting in a reduced parasite growth rate over the asexual blood stage[336].
PF3D7_0629500	Amino acid transporter, putative		RMgm-832	PBANKA_1128300	Gene disruption successful however the phenotype has not been described*.
PF3D7_0715800	Drug/metabolite exporter, drug/metabolite transporter		RMgm-1388	PBANKA_1422100	Gene disruption successful and no differences were observed when compared to wild type[424].
PF3D7_0715900	Zinc transporter, putative		RMgm-1373	PBANKA_1422200	Gene disruption successful, no differences observed over asexual blood stage, reduced exflagellation, reduced ookinete numbers, reduced sporozoite numbers, delayed transmission <i>in vivo</i> [424].
<i>Plasmodium</i>	Product description	Invadome	RMgmid	Rodent malaria	Modification attempted in rodent malaria parasites

<i>falciparum</i> gene ID				gene ID	
PF3D7_0801000	Plasmodium exported protein (PHISTc), unknown function		RMgm-708	PBANKA_1229000	C57Bl/6 mice infected with knockout parasites showed longer survival rates and a mild reduction in the growth rate of the asexual blood-stages when compared to wild type parasites[440].
PF3D7_0805300	Conserved Plasmodium protein, unknown	1	RMgm-444	PBANKA_1225300	Gene disruption was successful however no phenotype has been described[434].
PF3D7_0811200	Conserved protein, unknown function		RMgm-780	PBANKA_1426300	mCherry tagged parasites, however the localization has not been reported*.
PF3D7_0811300	CCR4-associated factor 1 (CAF1)		RMgm-318	PBANKA_1426200	<i>P. berghei</i> disruption was not successful The number of merozoites per schizont did not differ between <i>Pf</i> Δcaf1 and wild type parasites, however the growth rate was reduced. <i>Pf</i> Δcaf1 parasites resulted in aberrant expression of merozoite proteins such as SUB1, SERA5, EBA175 and GAP45[441]
PF3D7_0824400	Nucleoside transporter 2 (NT2)		RMgm-1392	PBANKA_0706200	Gene disruption successful, no differences observed over asexual blood stage[424].
PF3D7_0828800	GPI-anchored micronemal antigen (GAMA)	1	RMgm-95	PBANKA_0701900	<i>P. falciparum</i> orthologue is refractory to gene knockout[442]. PbΔpsop9 showed reduced numbers of oocysts and reduced number of salivary gland sporozoites[341].
PF3D7_0830500	Tryptophan/threonine-rich antigen (TryThrA)		RMgm-1252	PBANKA_0701000	Assigning orthology between genes of different species is difficult as multiple genes near/in sub-telomeric regions encode them.
PF3D7_1027800	60S ribosomal protein L3 (RPL3)		RMgm-1567	PY17X_0513000	GFP-tagged protein shows expression in the cytoplasm and the periphery of the parasite[443].
PF3D7_1033200	Early transcribed membrane protein 10.2 (ETRAMP10.2)		RMgm-726	PBANKA_0517000	Gene disruption successful, no differences observed over asexual blood stage.
PF3D7_1113000	Conserved Plasmodium protein, unknown	1	RMgm-105	PBANKA_0934600	Gene disruption attempts were successful, however a phenotype has not been described*
PF3D7_1116800	Heat shock protein 101, (HSP101)		RMgm-1327	PBANKA_0931200	<i>P. berghei</i> parasites with mutant HSP101 showed reduced growth rate (parasitemia) in mice[444].
PF3D7_1129900	Transporter, putative		RMgm-1368	PBANKA_0918300	Gene disruption resulted in a reduction in the parasite multiplication rate at the asexual blood stage of infection[424].
PF3D7_1222700	Glideosome-associated protein 45 (GAP45)	1	RMgm-776	PBANKA_1437600	GAP45 is considered vital for merozoite invasion of erythrocytes. <i>Pb</i> ΔGAP45 zygotes failed to develop into ookinetes[352].
PF3D7_1228600	Merozoite surface protein 9 (MSP9)	1	RMgm-1536	PY17X_1445800	GFP-tagged MSP9 shown to be expressed in the cytoplasm of infected erythrocytes[443].
PF3D7_1249800	THO complex subunit 2, putative (THO2)		RMgm-443	PBANKA_1462700	Gene disruption was successful however; a phenotype has not yet been reported*
PF3D7_1320600	Ras-related protein Rab-11A (RAB11a)		RMgm-298	PBANKA_1418900	GFP-tagged Rab-11A showed an apical localisation in merozoites partially co-localising with MSP1[445].
PF3D7_1345100	Thioredoxin 2 (TRX2)		RMgm-918	PBANKA_1358000	<i>Pb</i> ΔTRX2 parasites showed delayed blood-stage growth in mice[429].
PF3D7_1349300	Tyrosine kinase-like protein (TKL3)		RMgm-1221	PBANKA_1362100	Gene disruption was successful and no difference in growth rate was observed between transgenic parasites and wild type[336].
PF3D7_1447900	Multidrug resistance protein 2 (heavy metal transport family) (MDR2)		RMgm-1165	PBANKA_1311700	mCherry tagged MDR2 showed weak localisation in blood-stages and ookinetes and the plasma lemma of merozoites. Reduced oocyst and sporozoite formation in <i>Pb</i> ΔMDR2[425].
PF3D7_1460600	Inner membrane complex sub-compartment protein 3, putative (ISP3)	1	RMgm-962	PBANKA_1324300	Gene disruption was successful and showed no difference in asexual blood stage or gametocyte or ookinete/oocyst or liver stage when compared to wild type parasites. GFP-tagging of ISP3 showed localisation to schizonts and merozoites[360].
PF3D7_1466400	Transcription factor with AP2 domain(s) (ApiAP2)		RMgm-398	PBANKA_1329800	Gene disruption was successful resulting in oocysts development that lacked sporozoites. GFP tagged ApiAP2 showed expression in nuclei of oocyst and salivary gland sporozoites[446].
PF3D7_1471100	Exported protein 2 (EXP2)		RMgm-921	PBANKA_1334300	<i>Pb</i> ΔEXP2 showed reduced hepatocyte infectivity of sporozoites and unable to result in a blood-stage infection in mice[447].
PF3D7_1474900	Trailer hitch homolog, putative (CITH)		RMgm-358	PBANKA_1301300	GFP-tagged CITH showed expression in blood stage parasites. No difference in asexual blood stages growth rate between <i>Pb</i> ΔCITH and wild-type parasites[448].

A total of 239 (64%) targets were either considered members of the invadome or for which genetic manipulation of their *P. berghei* orthologues attempted. These represent additional information that could be considered when selecting targets for further evaluation as potential vaccine candidates. Interestingly, a small subset of these targets, N=59 (25%) was described as “conserved targets” of unknown function. I compared the degree of polymorphism between these targets and the current blood stage vaccine candidates under evaluation in clinical trials. These were MSP-1, -2, -3, AMA1, EBA175, RESA, GLURP, SERA-5, RH5 and PFF0165c. As shown in Figure 3.13, the blood stage vaccine candidates had a significantly higher dN/dS ratio when compared to these 59 targets (median dN/dS in vaccine candidates (N=10) versus conserved targets (N=59): 7.675 versus 2.360; p-value <0.0001).



**Figure 3.13: A comparison of the ratio of non-synonymous to synonymous SNPs (dN/dS) in vaccine candidate and unstudied targets identified as putative surface proteins.** Comparison of the dN/dS ratio in the genes encoding 10 blood stage vaccine candidates (blue) and targets described as being conserved and of unknown function.

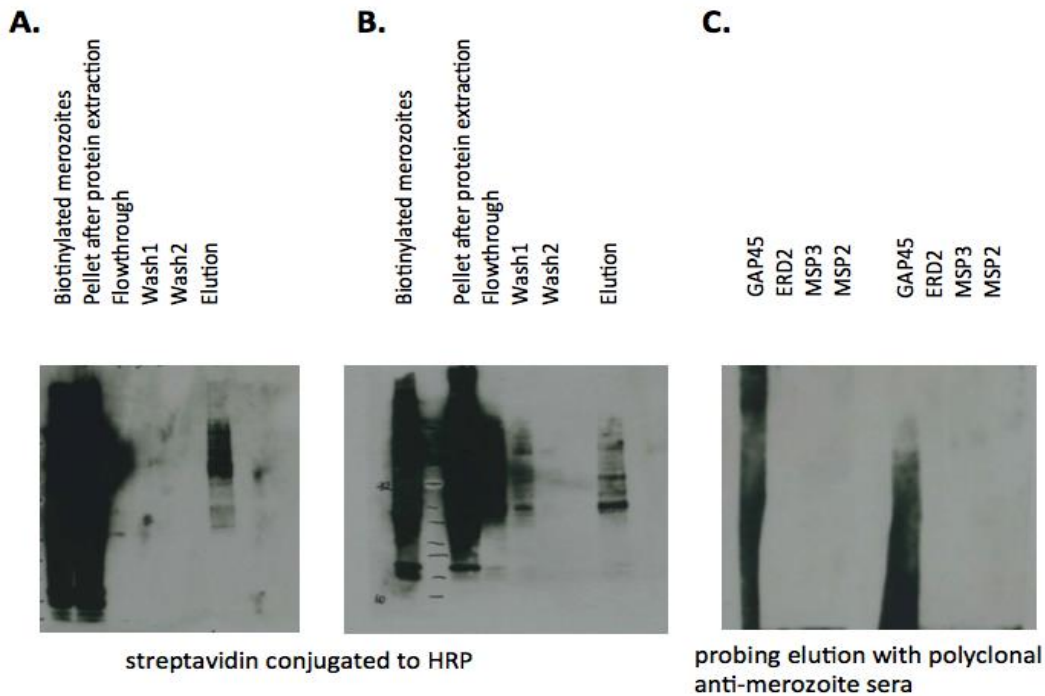
The list of the remaining 135 (36%) (Figure 3.12) putative surface merozoite antigens identified in both biological replicates is available in Appendix 3.5. In this list, 10 (7%) are predicted to have an extracellular/membrane localisation and the majorities 118 (87%) have no predicted localisation. Among those predicted to have an extracellular or membrane localisation included transporters such as: triose phosphate transporter (PF3D7\_0508300), inorganic anion antiporter (PF3D7\_1471200;SulP), sodium-dependent phosphate transporters (PF3D7\_1340900:PiT) and CorA-like  $Mg^{2+}$  transporter (PF3D7\_1304200.2). Interestingly, 67 (50%) of these targets are termed as “conserved plasmodium protein, unknown function” or “conserved plasmodium membrane protein, unknown function”. A small proportion of the targets were predicted to localise to the nucleus and cytoplasm, 2 (1.5%) and 4 (3%) respectively. This list may also contain potential novel targets of protective antibodies or anti-malaria drug targets.

### ***3.5.7. Optimising the merozoite surface-biotinylation experiments***

A second approach was attempted to identify merozoite surface proteins that relied on the following steps: 1) a cell-membrane impermeable reagent was added to merozoites to covalently attach biotin to the N-terminus of a protein or the free amine group on lysine residues of proteins on the surface, 2) biotinylated proteins were extracted and 3) affinity purified using a streptavidin column. Several protocols were attempted and I monitored the specificity of the reaction by probing the fractions and eluates using the following primary antibodies: streptavidin conjugated to HRP, anti-ERD2 and anti-MSP3 antibodies.

The streptavidin-HRP antibody was used to monitor the biotinylation reaction and to confirm affinity purification of biotinylated proteins. Anti-MSP3 sera was used as a positive control as MSP3, a peripherally associated merozoite surface protein, should ideally be biotinylated and detectable in the eluate. Anti-ERD2 was used as a negative control as it is an endoplasmic reticulum resident protein and therefore

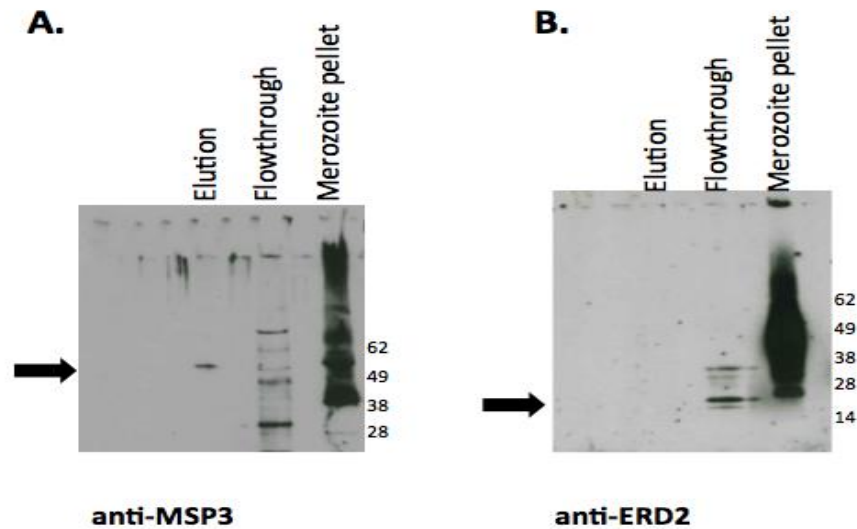
ideally inaccessible to the biotinylation reagent and therefore absent in the eluate following affinity purification. As shown in Figure 3.14A and B, biotinylated merozoite proteins were present in the eluate following extraction with 8M Urea or 4% SDS lysis buffer when probed with streptavidin-HRP. However, as shown in Figure 3.14C, known merozoite surface or membrane proteins such as MSP3 and MSP2 were not evident in any of the eluates. This indicated that merozoite proteins were biotinylated, however it was not possible to confirm whether these were surface or internal proteins. I therefore attempted a second protein extraction protocol aimed at enriching for membrane proteins.



**Figure 3.14: Merozoite surface biotinylation, extraction and western blot.** Biotinylated merozoite proteins extracted with A) 8M Urea and B) 4% SDS lysis buffer and probed with streptavidin-HRP. C) Probing of eluates from affinity purification using streptavidin column with the merozoite surface/membrane protein sera anti-GAP45, anti-MSP2 and anti-MSP3 and the internal negative control anti-ERD2. Figure A) and B) confirm the presence of biotinylated proteins. However on probing with positive and negative control sera, no antigen-specific bands were visible (C, limiting the ability to confirm the specificity of the biotinylation reaction).

Due to the absence of known merozoite surface proteins in the eluate from extraction with 8M urea and 4% SDS buffer, I attempted a second extraction buffer,

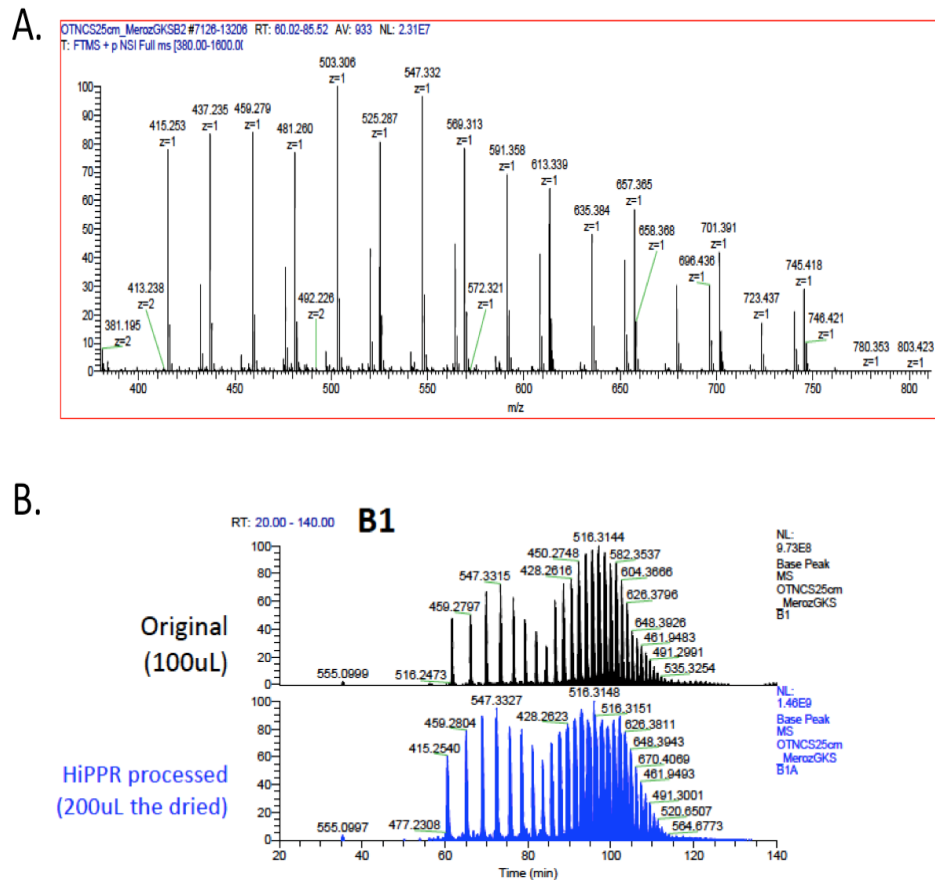
the non-ionic detergent 2% Triton X-100 that is commonly used to lyse cells and permeabilize cell membranes. As shown in Figure 3.15A, the *Pf*MSP3 positive control was detected in the eluate following affinity purification as expected. Similarly, *Pf*ERD2 the internal protein was absent in the elution and present in the flow through as shown in Figure 3.15B. The eluate from two biological replicates of the surface biotinylated merozoites in which *Pf*MSP3 was detectable and *Pf*ERD2 was absent was submitted for mass-spectrometry identification.



**Figure 3.15: Successful merozoite surface biotinylation, extraction and western blot.** Biotinylated merozoite proteins extracted with 2% Triton X-100. Probing of eluates from affinity purification using A) anti-MSP3 and B) the internal negative control anti-ERD2. As expected, MSP3 was detected in the eluate while ERD2 was detected in the flowthrough.

Despite detecting MSP3 in the eluate it was not subsequently identified by mass-spectrometry. As shown in Figure 3.16A, the MS-spectra from analysis of the eluates showed an abundance of “peptides” with a single positive charge ( $z=1$ ), suggesting that these were actually Triton X-100. These abundant ions likely masked the ability to detect the less abundant protein peptides that were of interest. Trypsin treated proteins generate peptides with at least two charges ( $z=2$ ) from the N-terminal end of a peptide and the positive charge from either the lysine or arginine residue that determines the specificity of the cleavage site. This feature is

used as a filter to select peptides for mass-spectrometry identification. I attempted to remove the excess detergent using the commercially available HiPPR (High Protein and Peptide Recovery) detergent removal spin column kit. As shown in Figure 3.16B, the mass-spectra profile obtained after detergent removal attempt was identical to the original sample. Therefore no data on merozoite surface biotinylated proteins was available for analysis. These experiments were not continued further due to time constraints.



**Figure 3.16: The MS-spectra obtained from analysis of eluates from merozoite surface biotinylation experiments.** A) The MS-spectra shows an abundance of peptides with a single charge ( $z=1$ ), as opposed to multiple charges ( $Z > 2$ ) that are characteristic of trypsin generated peptides. B) Efforts to remove contaminating Triton X-100 from eluate were unsuccessful as the mass spectra profiles are similar after HiPPR treatment (blue) when compared to untreated samples (black). HiPPR: High Protein and Peptide Recovery.

### 3.6. Discussion

#### *The merozoite proteome is largely under-studied in the context of NAI*

Although protective antibodies to blood-stage antigens are acquired following natural exposure to *P. falciparum* malaria [127], a surprisingly small portion of the merozoite proteome has been evaluated in this context. Mining data obtained from PlasmoDB indicated the presence of many potential merozoite surface proteins. As discussed in section 3.1 of this chapter, 550 and 571 genes whose transcriptional profiles are elevated in the late trophozoite-schizont stage of the erythrocytic life cycle are predicted to encode antigens with an N-terminal signal peptide or at least one transmembrane domain respectively. These represent potential surface-exposed antigens that may be targets of protective antibodies. Only a small proportion (N=157, (20%)) of these targets have been evaluated as potential targets of protective immunity. Thus the potential of most of these proteins as vaccine candidates is unknown[146, 263, 265, 267, 271, 292]. I therefore employed complementary proteomic approaches to comprehensively identify proteins on the surface of merozoites in order to generate a rich resource of potential targets of protective humoral immunity for further analysis.

#### *Identification of novel putative merozoite surface proteins*

Surface biotinylation was attempted but was abandoned due to difficulty with protein solubilization. In contrast, surface shaving combined with multiple bioinformatic analyses identified a total of 374 proteins that are likely to be either on or associated with the surface of the merozoite. The validity of this list as a source of potential targets is supported by multiple observations. First, it included multiple known merozoite surface proteins such as the GPI-anchored 6-cys proteins *Pf12* [294] and *Pf38* [294], the peripherally associated surface proteins such as GLURP, MSP-8, -9, -10 [407] and *Pf41* [294] and plasma membrane embedded proteins such as ROM1 and ROM4 [407]. Second, I identified an overlap between the identified proteins and the invadome (Figure 3.12), which is a list of genes, proposed to be important/essential for merozoite invasion of erythrocytes based on



coordinated transcriptional patterns. Lastly, I observed that the putative surface proteins I identified were significantly more polymorphic when compared to intracellular proteins, suggesting that they were under immune selection[290-292]. Interestingly, a subset of these proteins' orthologues has been identified in rodent malaria and genetic disruption of these genes in *P. berghei* had a negative impact on parasite growth (Figure 3.12 and Table 3.3).

However, only 16% (N=59) of the 374 proteins I identified as likely surface proteins have been analysed either in population studies to identify correlates of immunity or progressed to testing in clinical trials. These included the 6-cys proteins *Pf41*, *Pf38* and *Pf12*, and the surface proteins MSP-9, -10, GLURP that have been tested for antibody reactivity with sera from Kenyan [146] and Papua New Guinean children [147]. One of the identified targets, GLURP, is a component of the multi-subunit GMZ2/alum vaccine alongside MSP3 that has undergone phase I and II clinical trials showing good safety, tolerability and immunogenicity in naïve adults [449], malaria-exposed adults [450] and children [216]. As expected, 84% (N=315) of the targets I identified have not been tested in the context of NAI, and no information is available on the function or cellular localisation for most of these proteins (N=135 (36%). This list therefore represents a rich resource of novel potential targets of protective antibodies or anti-malarial drugs. My analysis ultimately yielded two lists of putative merozoite surface proteins; 1) targets for which some published literature is available (e.g. roles in erythrocyte invasion or a phenotype in the rodent malaria orthologues) the majority of which have not yet been evaluated as potential vaccine candidates and 2) targets for which no known information is available representing true novel potential targets. Both sets of proteins warrant further evaluation for reasons outlined below.

#### ***Putative surface proteins with potential as vaccine or drug targets based on literature***

Sixty four percent (N=239) of the putative merozoite surface proteins that I identified have either been proposed to play important roles in erythrocyte invasion

and parasite survival, either as members of the invadome [258] or based on genetic disruption experiments in *P. berghei* orthologues’.

Members of the invadome: Seventy (19%) of proteins I identified are encoded by genes whose transcriptional profiles showed differential expression on exposure to 20 parasite growth inhibitory compounds and are thought to be essential for erythrocyte invasion (termed the invadome) [258]. Antibodies against some of these targets could therefore be predicted to reduce or halt parasite growth altogether making them potential vaccine candidates. For example, the pantothenate transporter (*PfPAT*: PF3D7-0206200) plays a role in the uptake of vitamin B, a precursor for coenzyme A, which functions as an enzyme activator in metabolism of fatty acids, carbohydrates and amino acids [451]. This protein localises to the periphery of merozoites within schizonts [421] and *P. falciparum* blood-stage parasites grown in the absence of pantothenate were not viable [451, 452]. In addition, genetic studies where the *P. berghei* orthologue of PAT was disrupted, a defect in forming mature oocysts was observed as the female and male gametes failed to egress from host erythrocytes [431, 432]. As a result, naturally acquired or vaccine induced antibodies against *PfPAT* may have the potential to inhibit blood and transmission stages of the parasite. Additional examples of potential vaccine or drug targets include *PfCDPK1* (PF3D7\_0217500) that co-localises with MSP-1 [420]. *PfCDPK1* phosphorylates two members (MTIP and GAP45) of the actin-myosin motor complex that drives merozoite entry into erythrocytes. Inhibition of *PfCDPK1* resulted in the inability of merozoites to egress from schizonts halting erythrocyte invasion [420]. These may represent a new target of anti-malarial protective antibodies or chemical inhibitors.

*P. berghei* orthologues: Data was available on the *P. berghei* or *P. yoelii* orthologues of 210 of the putative surface proteins I identified (Figure 3.12 and Table 3.3). These included proteins in which attempts to knockdown the gene were not successful implying essential roles in the erythrocytic stage of the lifecycle [343, 345, 351, 422-426, 428, 429]. Interestingly, four of these are described as *P. falciparum* conserved membrane proteins of unknown function. In addition, for

some proteins for which genetic disruption was successful, a phenotype suggesting that targeting these proteins could reduce or halt parasite growth was reported [336, 341, 431, 432, 435, 438, 440, 441]. For example, genetic disruption of *P. falciparum* CCR4-associated factor 1 (CAF1), resulted in a reduced parasite growth rate, defects in merozoite egress from schizonts and aberrant localisation of EBA175 and GAP45 [441] among others. Due to these potential essential roles in parasite survival, antibodies or drugs against these targets are likely to have potent growth inhibitory activity making these potentially attractive vaccine or antimalarial drug targets. There was an overlap of 41 proteins that are proposed members of the invadome and for which genetic disruption in *P. berghei* has been attempted (Figure 3.11). These include proteins such as *PfPAT* and *PfCDPK1* that have been described above. Similarly, these are potential targets for therapeutic intervention.

Conserved proteins of unknown function: Encouragingly, 59 (25%) (Figure 3.12 & 3.13) of the proteins proposed to be members of the invadome and/or with *P. berghei* orthologues are referred to as conserved proteins of unknown function on PlasmoDB. Based on this PhD thesis, these might be proteins located on the surface of the merozoite. Interestingly, these targets were significantly less polymorphic than the current blood-stage vaccine candidates in clinical trials (Figure 3.13). These may represent ideal targets that may overcome the challenge of designing a vaccine that would be effective against genetically diverse parasites [453] and warrant their evaluation further as targets of protective antibodies. For example, clinical trials using the following candidates, RTS'S (CSP) and Combination B (MSP-1, -2 and RESA), have been shown to induce strain-specific responses that ultimately resulted in low vaccine efficacy [171, 222]. This was attributed to clinical episodes of malaria in vaccinated individuals resulting from parasites that were diverse at the candidate proteins' genetic loci. For these and other polymorphic vaccine candidates, a multivalent (vaccine consisting of multiple alleles of the same antigen) or a multi-subunit (vaccine consisting of multiple distinct antigens) approach may be better and is under evaluation [380]. An alternative approach would be to target a relatively conserved antigen with the expectation that vaccine-induced responses

would be effective across diverse parasite isolates. This has been demonstrated for the conserved target reticulocyte binding protein homologue 5 (RH5), in which only 5 non-synonymous SNPs have been reported at over a 10% frequency in 290 clinical isolates from diverse geographical locations [454]. Antibodies raised against the 3D7 variant of RH5 showed broad inhibitory activity against diverse *P. falciparum* lab isolates and strains [454, 455] in growth inhibition *in vitro* assays. In subsequent vaccination followed by challenge studies, Aotus monkeys vaccinated with the Pf3D7 variant of RH5 and challenged with the virulent heterologous FVO strain showed reduced parasitemia and ability to self-cure when compared to control animals[456]. This demonstrated the *in vivo* vaccine induction of responses that were protective against heterologous strains, showing the utility of using a conserved vaccine candidate.

### ***Novel putative surface proteins for therapeutic intervention***

A large proportion of the *P. falciparum* protein coding genes have unknown functions on PlasmoDB and not surprisingly, 18% (N=67) of putative surface proteins I identified fall within this category (Figure 3.12). These include predicted transporters whose functions have not been confirmed and proteins described as being conserved and of unknown function. Additional studies are needed to begin to understand the functional roles of these proteins. These could include studies that evaluation the transcriptional profiles of these proteins in parasite exposed to the 400 diverse drug-like molecules available in the Malaria box (available from Medicines for Malaria Venture, MMV) [457, 458]. Similarly, high-throughput screening of these novel targets for naturally acquired antibody responses could be evaluated. For example, the use of a phage-display library [459, 460] expressing all the proteins of unknown function that I identified could subsequently be screened for recognition using sera from individuals exposed to *P. falciparum*.

### ***Study limitations***

The above approaches have various limitations shared by all proteomic studies, the most common of which is the identification of contaminant proteins. I monitored the

specificity of the surface-shaving experiment by observing the release of the peripherally associated protein MSP3 in the enzyme treated merozoites while the internal protein ERD2 was retained. Despite this, I observed the presence of a small proportion of proteins (less than 10%) predicted to be nucleus-associated, cytoplasmic or mitochondrial. These are likely to have been detected due to merozoite lysis or damage during the schizont filtration or incubation steps resulting in the release of intracellular targets. Additional replicate data would have been beneficial in differentiating between true targets and contaminant proteins.

Contaminants in proteomic studies are common as illustrated in a recent review on the application of the surface-shaving technique to bacterial and eukaryotic pathogens, which showed a range of between 6-80% of proteins identified as being localised to the cytoplasm [414]. Nevertheless, I cannot exclude the possibility that the predicted intracellular targets I identified are in fact “moonlighting proteins” [417]. These proteins, which are often enzymes, lack the typical motifs that determine their extracellular localization such as N-terminal signal peptides or transmembrane domains. However, they have been shown to be present on the cell surface of pathogens and play dual roles that include their intracellular enzymatic function as well as a non-catalytic role when they translocate to the cell surface. Examples include the intracellular enzyme GAPDH (glyceraldehyde-3-phosphate dehydrogenase) that was shown to be highly immunogenic in systematic candidiasis and subsequently shown to be also located on the surface of *Candida albicans* as a ligand binding to fibronectin and laminin [461]. Although difficult to validate in this study, this is a potential explanation for the observation that intracellular or cytosolic enzymes or proteins were classified as surface proteins in my study. Interestingly, antibodies against intracellular proteins have been reported to be associated with protection from malaria in some studies [265]. A second limitation was the inability to generate useful data from the complementary merozoite surface biotinylation approach. As I observed that the non-ionic detergent Triton X-100, was capable of extracting the surface protein *Pf*MSP-3, attempts with similar

detergents that are compatible with mass-spectrometry identification should be evaluated [462].

Despite the above limitations, my study provides strong evidence for a set of novel proteins that are potentially located on the surface of merozoites. Additional experimental approaches would help to further validate these observations. Confirmatory assays could involve generating recombinant forms of the novel antigen, raising polyclonal sera and conducting immuno-fluorescent assays (IFAs), to map their localisation [294, 419, 421, 463]. This approach has been successfully used to show co-localisation of *Pf*12 and *Pf*41 with MSP-1<sub>19</sub> on the surface of free merozoites [383]. Alternatively, transgenic parasite lines could be generated in which an epitope tag such as the c-myc or HA-tag, or a fluorescent proteins such as the green fluorescent protein (GFP) or mCherry is added to the C-terminus of the protein of interest, in order to enable localisation using fluorescent microscopy. These approaches have been used to localise a putative sugar transporter (PY17X-0823700;ortholog of PF3D7-0919500) to the surface of sporozoites [301]. Similarly, the localisation of *P. berghei* RAP2/3 to the rhoptry organelles on merozoites and sporozoites was achieved using a c-myc tagged transgenic line [345].

## **Conclusion**

In summary, I identified 374 putative surface proteins using merozoite surface shaving, the majority of which have unknown functions and have not been studied in the context of naturally acquired immunity. Data on the 30% of proteins for which some published information is available suggest that these proteins may have important roles in erythrocyte invasion or be essential for parasite survival. This provides a rich resource for further study for the identification of novel drug and vaccine candidates. In line with evaluating their utility as targets of protective antibodies, I have since selected a subset of these proteins and shown that they are reactogenic in serum collected from adults living in a malaria endemic region of Tanzania. In addition, antibody levels to some of these targets such as *Pf*PAT

(PF3D7-0206200) and a putative amino acid transporter (PF3D7-0629500) are associated with a reduced number of clinical episodes of *P. falciparum* malaria. The details of the down-stream selection criteria of a subset of these antigens for further analysis, and their subsequent analysis in immuno-epidemiology studies are presented in Chapter four and five of this thesis.

## CHAPTER 4

### **Novel *Plasmodium falciparum* merozoite antigen selection and characterization for antibody reactivity**

#### **4.0 . Introduction**

The -omics era has enabled the rapid generation of large data sets that provide an unprecedented opportunity for the discovery of novel vaccine candidates, drug targets or essential host or pathogen determinants of infection and survival. However, the subsequent systematic evaluation required for the large numbers of newly identified candidates can be labour intensive and daunting. Down-selection criteria can help to focus attention on targets with the highest probability of utility. For example, recently a forward genetic screen employing RNA interference (RNAi) was used to identify host determinants of malaria infection [464]. Although over 350 proteins that appeared to be involved in erythrocyte invasion by *P. falciparum* were identified in the initial screen, subsequent validation experiments were focused on the 42 proteins that shared similarities with known invasion receptors (members of the human blood group antigens). This analysis led to the identification of CD55 as an essential receptor for *P. falciparum* invasion [464].

In the context of drug and vaccine identification in *P. falciparum*, comparative genomics [253], proteomics [254, 255] and transcriptional profiling [256-258] of parasites have provided valuable information to use as down-selection criteria for new malaria vaccine targets. Datasets that are currently publicly available include; 1) the genome sequences of 2512 *P. falciparum* isolates under the Pf3K project [465] to provide gene diversity data, 2) population genomic analyses of 65 Gambian isolates for the identification of genes under balancing selection [253] and hence may be exposed to the human immune system, 3) proteomic analyses of sporozoites, trophozoites, merozoites and gametocytes obtained from the 3D7 and NF54 strains [254, 255] and 4) transcriptional profiling of the 3D7 and HB3 strains in the intraerythrocytic developmental stages [259, 260], gametocytes and mosquito



salivary gland sporozoites stages [260] to identify the stages in which candidates are expressed. These data sets continue to be harnessed when selecting targets for laboratory evaluation. For example, to identify *P. falciparum* genes with important roles during the erythrocytic life cycle, changes in gene expression throughout the asexual stages was measured following exposure to 20 chemical compounds known to inhibit parasite growth [258], in order to identify co-regulated genes that may function in the same pathways. A total of 3125 genes showed over a three-fold increase or decrease in gene expression compared to uninhibited parasites. From this datasets, targets likely to play a role in merozoite invasion of erythrocytes were identified by; 1) comparing the sequence homology between newly identified targets and orthologue genes in 210 sequenced genomes involved in conserved functional pathways, 2) predicting protein-protein interactions using available yeast-two hybrid interactome data and 3) utilizing transcriptional profiling data on lab and field isolates to identify genes up-regulated during the late blood stages. Probabilistic networks were generated from this data to cluster the identified targets [258]. Thereafter, the authors selected the targets that clustered around 27 known merozoite targets involved in erythrocyte invasion. This resulted in a list of 418 targets (termed invadome or invasion-related genes), 263 (63%) of which were described as hypothetical proteins of unknown function. A subset of predicted invasion-related genes were selected for sub-cellular localization and they demonstrated for the first time, the surface localisation of PF3D7\_1036000 now known as MSP11, and two conserved *Plasmodium* proteins namely PF3D7\_0308300 (PFC0355c) and PF3D7\_1115800 (PF11\_0166) [258].

These publicly available datasets have been harnessed for the identification of potential vaccine candidates in five other studies. These include two studies that selected targets for evaluation only from publicly available datasets [263, 264] and three studies that generated additional proteomic data from sporozoite [301] and asexual stages [299, 316] prior to evaluation using available datasets. In each of these studies one or more of the following criteria were used to prioritize targets for laboratory evaluation: 1) elevated mRNA transcription at the schizont stage (40-48

hpi) or 2) identified as a member of the invadome as has been described above[258] or 3) presence of a signal peptide or 4) one or more transmembrane domains or GPI-anchor and 5) have homologues present in different organisms of known function. In the two non-proteomic studies, a total of 113 unique targets were selected. One study subsequently identified 7 proteins, MSP3.5, MSRP2, ETRAMP11.2, ETRAMP14.1, RALP1, StAR-related lipid transfer protein and a conserved membrane protein (PF3D7\_1459900) as immunogenic for the first time [263]. The second study characterised the rhoptry associated adhesin (RA) as a protein that translocates to the merozoite surface and interacts with erythrocytes [264]. Antibodies generated against this target showed modest invasion inhibitory activity [264]. The three large-scale proteomic studies identified between 33 and 423 sets of proteins. They subsequently applied a range of one or more of the criteria described above and narrowed down to 2-4 proteins per study, for further laboratory evaluation. These resulted in identifying novel proteins on the surface of parasite-infected erythrocytes namely PF3D7\_0310400 (PFC0435w) and PF3D7\_0501200 (PFE0060w), now known as PIESP1 and 2 (parasite-infected erythrocyte surface protein 1 and 2) respectively [299]. Similarly, an uncharacterised antigen, PF3D7\_0919500 (a sugar transporter) was shown to localise to the surface of salivary gland sporozoites and for the first time, provided evidence that some components of the inner membrane complex (IMC) are surface exposed [301]. The last study demonstrated the secretion of four novel proteins during the erythrocytic stages of the life cycle with potential roles in immune evasion and host-pathogen interactions [316].

In this chapter, the target lists I generated in the previous chapters using proteomic approaches were evaluated and down-selected using publicly available databases, peer-reviewed publications and *in-silico* prediction algorithms in order to prioritize novel targets for further characterisation. However, it differs from previous efforts to identify potential targets of naturally acquired antibody responses in the following ways. I focused on generating a list of immunogenic merozoite targets as well as proteins localised to the surface of merozoites using proteomic approaches

prior to employing a down-selection criteria. This is in contrast to selecting targets directly from the published *P. falciparum* genome based on the same criteria as has been employed in studies by Fan *et al* [263] and Crompton *et al* [265]. This was expected to result in a higher rate of target identification compared to the average rate of 20% obtained with the previous efforts [263, 265, 271]. Secondly, the majority of targets I selected were either conserved or of limited polymorphism. This has potential implications on vaccine efficacy across different geographical locations where genetically diverse parasites isolates are present. Similarly, previous large-scale studies did not consider the level of polymorphism at each gene loci selected as a criterion for inclusion or exclusion.

#### **4.1. Rationale**

Using the proteomic approaches described in chapter 2 and 3 of this thesis, I identified a set of antigens that are potential targets of naturally acquired antibodies (N=210) and putative surface merozoite proteins (N=374) respectively. I then used several selection criteria to narrow down on antigens for further laboratory characterisation as potential targets of protective humoral immunity. These included selecting targets common to both experimental approaches and predicted to have an N-terminal signal sequence and/or a transmembrane domain(s). In addition, I compared the selected targets with publicly available data on: i) balancing selection; ii) potential roles in erythrocyte invasion, iii) have orthologues in rodent malaria species for which genetic disruption has been attempted and iv) studies of naturally-acquired immunity, in order to identify those poorly or never previously studied.

## 4.2. Objective

The overall objective was to select and evaluate a set of novel *P. falciparum* merozoite antigens as targets of naturally acquired antibody responses.

## 4.3. Specific objectives

- To select a sub-set of novel antigens identified in proteomic studies for further characterisation as targets of naturally acquired immunity (NAI).
- To express recombinant forms of the novel antigens selected above for evaluation of reactivity with human antibodies obtained from naturally exposed individual.

## 4.4. Methods

### 4.4.1. Selection of novel antigens

Using the proteomic approaches described in chapter two and three of this thesis; I identified 210 antigens in the 3D7 immunoblot assays and 374 targets in two biological replicates of 3D7 merozoite surface shaving experiments. To narrow down on which of these targets to evaluate in the context of NAI, the following down-selection procedures were applied. In the first step, targets that were identified in all the three data sets that I had generated, 2D-immunoblots and the two biological replicates of the surface-shaving experiments were selected. In the second step, targets identified in the 2D-immunoblots that had a predicted N-terminal signal peptide and/or at least one transmembrane domain were further shortlisted. Similarly, in the third step, targets identified in both surface-shaving biological replicates and that had a predicted N-terminal signal peptide and/or at least one transmembrane domain were chosen. Within this subset (N=222), I down-selected further using the following criteria 1) candidates that had been evaluated for reactivity with naturally acquired antibodies in less than 3 studies or not at all, 2) those with elevated maximum transcriptional profiles in the late trophozoite or schizont stages 3) those with evidence of balancing selection and 4) those that had a phenotype following genetic disruption in the respective *P. berghei* orthologue

(RMgmDB database). At the time of protein selection, phenotypic data on *P. berghei* reverse genetic studies now deposited on PlasmoGEM, was not publicly available.

#### ***4.4.2. Design of selected antigens for recombinant protein expression***

A total of 27 antigens were selected for recombinant protein expression using the Expi293 mammalian expression system (ThermoFisher) that is commercially available. This system is designed for scalable transient transfection of high-density cultures of human embryonic kidney 293 (HEK293) cells for recombinant protein expression[466]. For each of the 27 antigens selected, I obtained the Pf3D7 protein sequences from PlasmoDB and submitted these to signal peptide and transmembrane domain prediction using the (<http://www.cbs.dtu.dk/services/SignalP/>) & (<http://www.cbs.dtu.dk/services/TMHMM/>), algorithms respectively. To obtain only the predicted extracellular domains of the selected antigens, amino acids representing the N-terminal signal peptides and the transmembrane domains were excluded. In proteins with multiple transmembrane domains, the regions predicted to be extracellular were selected for recombinant protein expression. All potential N-linked glycosylation sites (NXS/T) were predicted using the (<http://www.cbs.dtu.dk/services/NetNGlyc/>) algorithm. The serine and threonine residues in predicted N-glycosylation sites were substituted with an alanine to prevent addition of glycans, which are thought to be absent in native *P. falciparum* proteins [467]. In proteins with no predicted N-terminal signal peptide, the first methionine was removed, as it was present in the final expression vector. Final amino acid sequences were submitted for gene synthesis and codon-optimisation for expression in human cells by GeneartAG, with, unique Not1 and Asc1 restriction sites inserted at the 5' and 3' end of the protein sequence respectively. This was done to clone each gene into a derivative of the pTT3 expression vector, in between a leader sequence to drive secretion of antigen and a rat Cd4 domains 3 and 4 tag followed by hexa histidine tag for protein purification. The leader sequence was the

N-terminal signal peptide that drives secretion of the mouse variable *kappa* light chain [468, 469]. The expression vector was obtained from the non-profit plasmid repository (<https://www.addgene.org/>). A schema of the sub-cloning strategy and the features of the expression vector is shown in Figure 4.1.

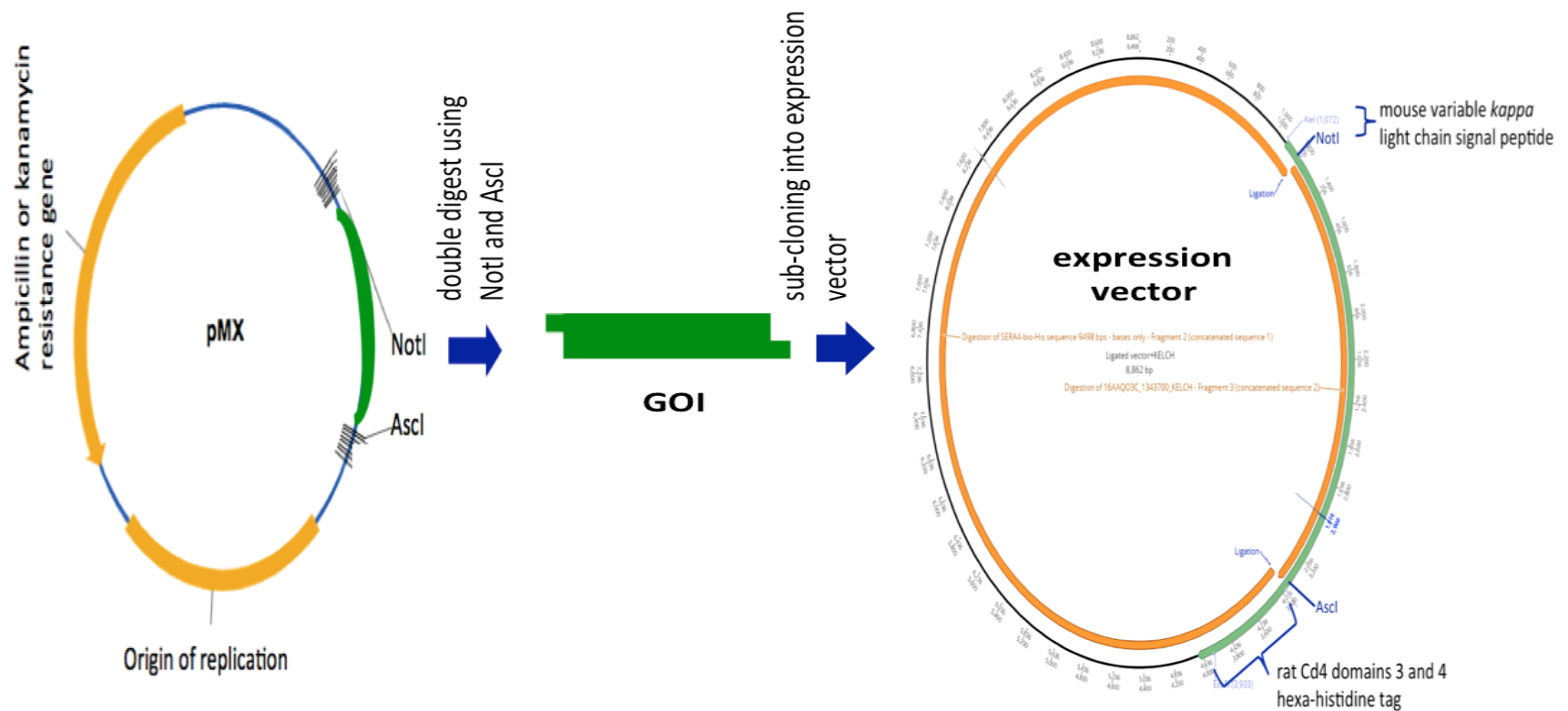
#### ***4.4.3. Sub-cloning and recombinant protein expression of novel antigens***

Codon-optimised gene constructs obtained from GeneartAG were received as a lyophilised plasmid vector with either the ampicillin or kanamycin resistance gene. To clone each gene into the expression vector, the following steps were taken: 1) excision of gene of interest (GOI) from backbone Geneart vector, 2) ligation of the GOI to expression vector and *E.coli* colony screening, 3) transfection of expi293F cells for recombinant protein expression and 4) protein purification from culture supernatant using the hexa-histidine tag. Prior to experimental procedures, *in silico* digestion was simulated using the Geneious R.9 software. This was used to predict the expected product size following restriction digestion and the expected protein sequence following successful cloning into expression vector. For each Geneart construct, I confirmed that *in silico* prediction of the expected protein matched the amino acid sequences requested.

##### ***4.4.3.1. Excision of GOI from the backbone GeneartAG vector***

Five micrograms (5µg) of lyophilised constructs from GeneartAG were resuspended using 50µl of TE buffer (10mM Tris, pH 8.0, 1mM EDTA) to a final concentration of 100ng/µl. These vectors also contain either a kanamycin or ampicillin resistance gene. To propagate these constructs, the plasmids were transformed (introduction of foreign DNA to bacterial cells) into competent Top 10 *E.coli* cells (Invitrogen) as follows: Top 10 cells stored at -80°C were thawed on ice and 2µl of resuspended plasmids added to 25µl of cells, gently mixed and incubated on ice for 30 minutes. Thereafter, cells were heat-shocked by incubation at 42°C for 30 seconds followed by incubation on ice for 2 minutes. Cells were resuspended in 250µl of SOC media at 37°C for 1 hour while shaking at 225 rpm before being resuspended in 300ml of LB broth supplemented with respective antibiotic overnight at 37°C. The heat-

shocking step is thought to change the fluidity of the cell membrane creating pores that allow exogenous DNA to enter cells[470]. After overnight incubation in LB-broth, *E.coli* cells were pelleted and the plasmid DNA extracted using the Qiagen maxiprep kit according to manufacturer's instruction. To excise the gene of interest flanked by Not1 and Asc1, a double digest using the respective enzymes was performed as follows: 0.5µl of Asc1 and Not1 (New England Biolabs (NEB)) were added to 2µl of the 10X cutsmart buffer (NEB), 10µl of respective plasmid DNA and top-up to 20µl using DNase/RNase free water. This reaction was incubated overnight at 37°C and then separated on a 1% agarose gel supplemented with red safe DNA dye at 100V until the loading dye line was 90% down the gel. Hyperladder I (Bioline) was used as the molecular size marker as it showed the separation of DNA whose sizes ranged 200-10000 base pairs. Separation of the GOI from backbone vector was visualised under UV light using the ChemiDoc MP system (Bio-Rad). Thereafter, the genes of interest that migrated at the expected base pair size were excised from the gel using a scalpel and forceps under observation using a UVP Chromato-vue transilluminator. The GOI was extracted using the QIAquick gel extraction kit (Qiagen) according to manufacturer's instruction. Extracted DNA was resuspended in 20µl in TE buffer and quantified using the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific).



**Figure 4.1: A schema of the *in silico* experiment in which the GeneartAG vector is double-digested to obtain gene of interest (GOI) and sub-cloned into expression vector.** The Geneart vector contains the NotI and AscI sites flanking the GOI. The same restriction sites were used to sub-clone the GOI downstream of the leader sequence and upstream of the Cd4-hexa histidine tag in the expression vector



#### 4.4.3.2. Ligation of the GOI to expression vector

The NEB calculator (<http://nebiocalculator.neb.com>) was used to design the parameters for ligation of the GOI to the expression vector using the formulae below.

Required mass insert (ng) = (desired insert/vector molar ratio) X (mass of vector (ng)) X (ratio of insert to vector lengths).

For example, using 50ng of the expression vector whose size was 6678 base pairs (bp) and a GOI of length 2191 bp at the recommended insert/vector molar ratio of 3:1, the quantity of insert DNA required was 49.21 ng. The ligation was done as follows: I combined 50ng of expression vector with 3 fold molar ratio of GOI and adjusted the volume of reaction to 10µl using distilled water. 10µl of 2X quick ligation buffer (NEB) was added to vector-DNA mix and 1µl of quick T4 DNA ligase was added and vortexed. The mix was allowed to incubate for 5 minutes at room temperature and stored at -20°C. This mix was then used to transform competent Top 10 *E.coli* cells as has been described in section 4.4.3.1 with the following changes. Instead of growing transformed cells in LB broth, these cells were plated on an LB-Agar plate supplemented with 100µg/ml of ampicillin and incubated overnight at 37°C. Following incubation, 8 colonies from each plate (corresponding to one GOI) were randomly selected and inoculated into 3ml of LB-ampicillin broth and grown overnight at 37°C. Plasmids were extracted from 1.5ml of each clone using the plasmid miniprep extraction kit (Promega) according to manufacturer's instructions. Extracted plasmids were digested using the Not1 and Asc1 restriction enzymes as was described in section 4.4.3.1. Two results were expected; 1) If the GOI were absent from expression vector, a single high molecular weight band at 6678 bp would be observed and 2) If the GOI was ligated into the vector, two bands were anticipated corresponding to the backbone vector and the GOI. Colonies showing the presence of the GOI were selected and grown in 400ml of LB-Broth to obtain sufficient quantity of target expression vector for transfection

of expi293F cells. These plasmids were extracted from *E.coli* using the Qiagen maxi prep kit (Qiagen), resuspended in TE buffer and quantified by NanoDrop.

#### ***4.4.4. Recombinant protein expression using Expi293F mammalian expression system***

To obtain target recombinant protein, expi293F cells were transfected with the expression vector using the commercially available expi293 expression system [466]. Expi293F cells stored in liquid nitrogen were thawed rapidly in a water bath at 37°C for 2 minutes with gentle agitation and resuspended in 30ml of pre-warmed expi293 expression media in 125ml Erlenmeyer flasks with vented caps. These cells were incubated at 37°C and 8% CO<sub>2</sub> in a humidified orbital shaker platform, rotating at 125rpm. The viability and total cell count was determined using an automated cell counter and monitored until the cell density was between 0.5-1 x 10<sup>6</sup> cells/ml. The culture was diluted to 3-5 x 10<sup>5</sup> viable cell/ml and monitored every two days until a maximal density of 4-6 x 10<sup>6</sup> viable cells/ml. Cell viability was monitored and cultures whose viability was lower than 90% was discarded. In addition, cells maintained in culture for longer than 3 months were discarded and a new batch of cells thawed. Prior to transfection, cell count and viability was determined and cells diluted to a 2.0 x 10<sup>6</sup> cells/ml with Expi293 media. For each antigen, a 25.5ml suspension culture at a 2.0 x 10<sup>6</sup> cells/ml density was transfected with 30µg of expression vector as follows. 30µg of DNA was diluted with 1.5ml of the Opti-MEM media and mixed gently. In a separate tube, 81µl of the cationic-lipid transfection reagent expifectamine 293 was diluted in 1.5ml of Opti-MEM media and incubated for 5 minutes at room temperature. The two reagents were then gently mixed making a total volume of 3ml and incubated for 20 minutes at room temperature. Thereafter, this was gently added to cells while swirling and incubated at 37°C and 8% CO<sub>2</sub> in an orbital shaker at 125rpm. After 16 hours of incubation, 150µl of enhancer 1 and 1.5ml of enhancer 2 was added and cells incubated for an additional 4 days. Thereafter, the cells were pelleted and the culture supernatant, which contained secreted recombinant protein, was stored at 4°C prior to purification.

#### *4.4.4.1. Purification of recombinant hexa-histidine tagged proteins*

Hexa-histidine tagged recombinant proteins were purified from culture supernatant using the Ni-NTA purification system. This system uses an agarose matrix cross-linked to nitrilotriacetic acid (NTA) that binds four  $\text{Ni}^{2+}$  per molecule, which has a high affinity ( $K_d=10^{-13}\text{M}$ ) for the hexa-histidine tag on recombinant proteins. Prior to purification, 30 $\mu\text{l}$  of 1mM of nickel chloride was added to supernatant, before binding to 1ml of Ni-NTA agarose overnight at 4°C while rotating. Following incubation, the agarose resin was pelleted and resuspended in native wash buffer (50mM  $\text{NaH}_2\text{PO}_4$ , 0.5M NaCl, 20mM imidazole, pH.8.0) and placed in a column for batch purification. The resin was washed five times with the wash buffer before elution using the native buffer (50mM  $\text{NaH}_2\text{PO}_4$ , 0.5M NaCl, 250mM imidazole, pH.8.0). Four-1ml eluted fractions were collected following incubation of resin with native elution buffer. These were pooled and concentrated to between 500-1000 $\mu\text{l}$  prior to separation on an SDS gel and quantification using the BCA kit according to manufacturer's instruction.

#### *4.4.5. Evaluation of the immunogenicity of novel antigens by standard ELISA*

Once purified recombinant proteins were obtained and quantified, I evaluated immunogenicity of these antigens by standard ELISA. For each antigen, 100 $\mu\text{l}$  of recombinant antigen was coated at a two-fold serial dilution of ranging from 128 $\mu\text{g}/\text{ml}$  to 0.06 $\mu\text{g}/\text{ml}$  on the wells of a Dynex 4HBX Immunolon plates. Recombinant antigen was diluted in coating buffer (15mM  $\text{Na}_2\text{CO}_3$ , 35mM  $\text{NaHCO}_3$ , pH 9.4) and coated overnight at 4°C. After overnight incubation, plates were washed four times in 1X PBS containing 0.05% Tween-20 (wash buffer) and blocked for 5 hours at room temperature with 1% skimmed milk (Marvel) diluted in PBS Tween 20 (Blocking buffer). Thereafter, plates were washed four times in wash buffer and 100 $\mu\text{l}$  of a pool of hyper-immune sera (PHIS) obtained from adults living in the malaria endemic region of Kilifi, Kenya and a pool of non-exposed sera from adults in Sweden (NEG) at a 1:1000 dilution were added as positive and negative controls respectively. Following an overnight incubation at 4°C, well were washed and incubated for 3 hours at room temperature with 100 $\mu\text{l}$  of HRP conjugated to

polyclonal rabbit anti-human IgG (Dako) at a 1:5000 dilution using block buffer. The wells were then washed four times and incubated at room temperature with 100µl of substrate development buffer (0.1M citric acid, 0.2M Na<sub>2</sub>HPO<sub>4</sub>, 4mg *o*-phenylenediamine dihydrochloride tablets (Sigma), 8µl hydrogen peroxide and 5ml distilled water). After 20 minutes of incubation in the dark, the reaction was stopped with 25µl H<sub>2</sub>SO<sub>4</sub> and absorbance read at 492nm.

#### **4.4.6. Statistical analysis**

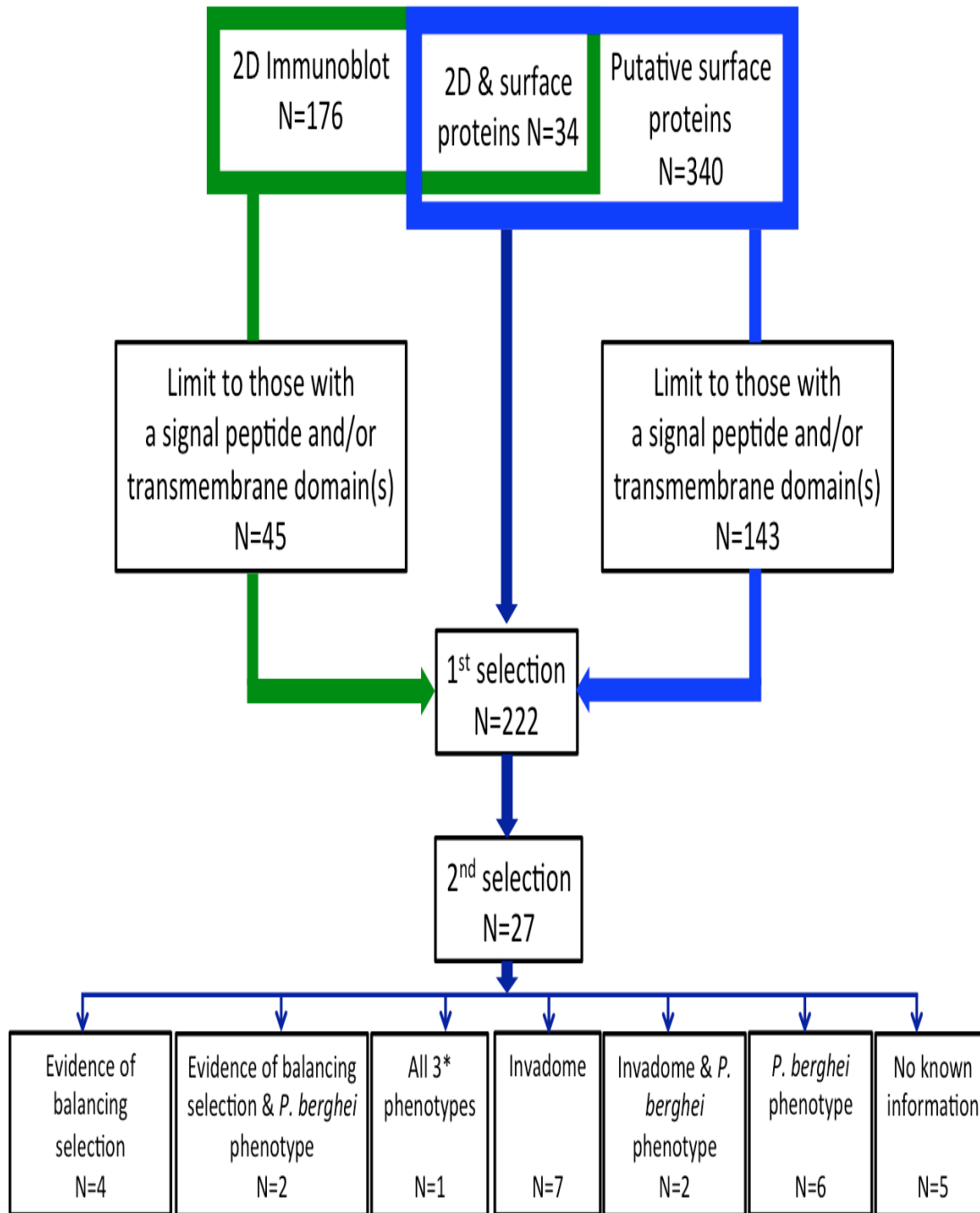
The non-parametric Mann-Whitney T-test was used to compare the Tajima D scores between selected antigens and non-selected targets. Graphpad Prism was used to generate the dose response immunogenicity curves for each antigen.

### **4.5. Results**

#### **4.5.1. Selection of novel merozoite targets for immunoprofiling**

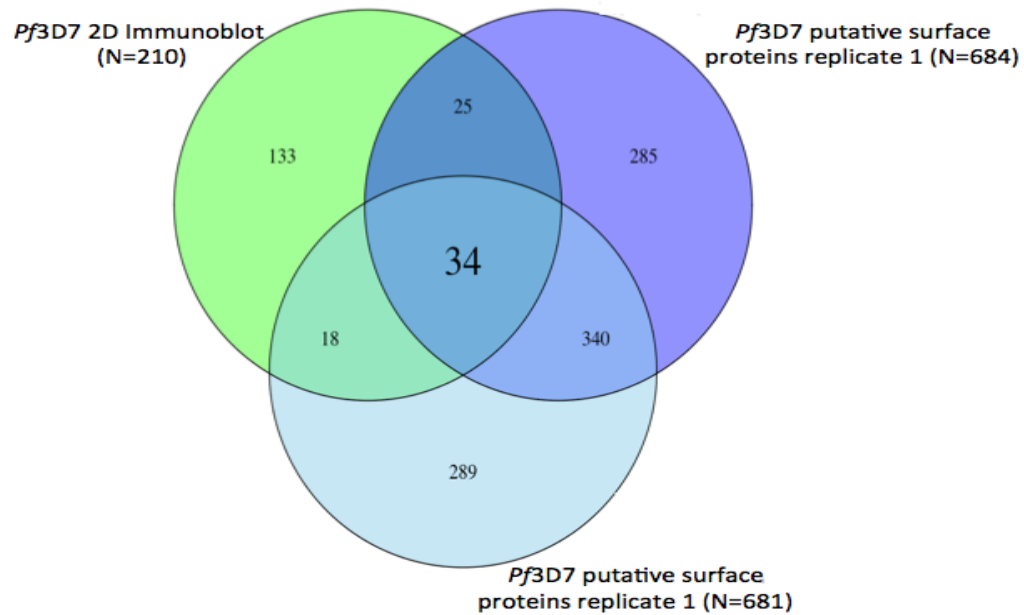
I used several criteria to select 27 novel *P. falciparum* antigens for immunoprofiling using the datasets I generated from the 2D immunoblots (N=210) and putative merozoite surface proteins (N=374). A summary of the selection procedure employed is shown in Figure 4.2, and is described in detail below.

To narrow down on the set of antigens from which to select targets for immunoprofiling, I first identified the proteins that were identified in all the three proteomic datasets I had generated. These were the targets identified in the 3D7 2D-immunoblot (N=210) and proteins defined as surface proteins from the two biological replicates (N=681 and 684 respectively). As shown in Figure 4.2 and 4.3, 34 targets were identified in all three data sets. These included known immunogenic targets such as GLURP, GAMA, KAHRP, MSP-8, MSP-9, PTEX150, RAP2 and MESA. In this subset of antigens, 8 (24%) were proteins described as conserved *Plasmodium* proteins of unknown function.



\*-Evidence of balancing selection, member of invadome and *P. berghei* phenotype

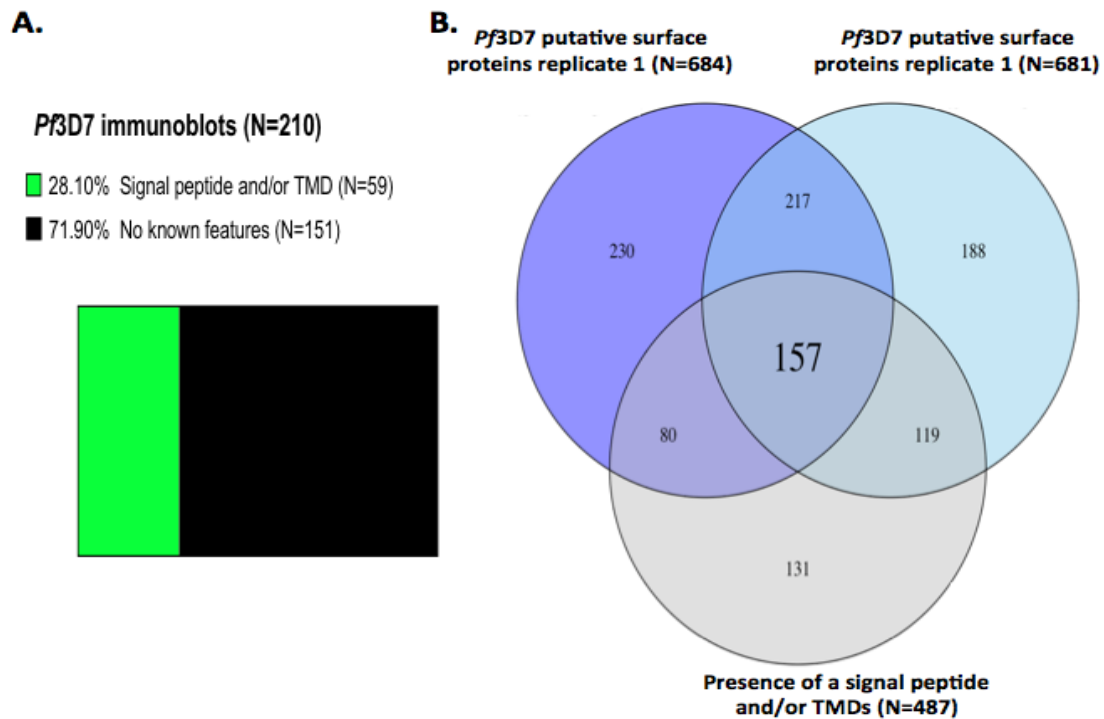
**Figure 4.2: An overview of the down-selection process employed to select 27 targets for immunoprofiling**



**Figure 4.3:** Figure showing the overlap between targets identified in the 3D7 2D immunoblots (green circle) and the two putative surface protein data sets (dark and light blue circles).

In a second and third selection, targets identified in the immunoblot or both surface shaving experiments that had a predicted signal peptide and or at least one transmembrane domain were selected and these corresponded to 59 and 157 antigens respectively (Figure 4.4 A & B, respectively). Fourteen of these targets had been previously selected in the first criteria. This resulted in a total of 222 antigens from which to select novel antigens from (Appendix 4.1). Additional criteria were used to select the targets for recombinant protein expression. From this list, 23 candidates were eliminated as they had already been selected for expression and analysis as part of a larger multi-centre cohort study. These targets included MSP-1, -3, -7, -8, -9 & -10, the SERA-3, -4 & -5 antigens, the 6-cys proteins *Pf41*, *Pf38*, *Pf12* & *Pf113*, AMA1, GAMA, MTRAP, GLURP, the rhoptry proteins RAP-1, -2 & RhopH3, PTEX150, ETRAMP10.2 & PF3D7\_0210600. An additional 40 targets were excluded as they have been/or are currently under evaluation in various groups as targets of naturally acquired immunity [265, 267]. The presence of these genes in the initial down-selection list is a useful validation of the initial gene list as potential sources of novel antigens.

These resulted in a list of 159 targets which I then compared against publicly available data on: i) evidence of balancing selection as indicated by a positive Tajima D score, when the genomes of 65 isolates in the Gambia were analysed [253], ii) proposed to be involved in erythrocyte invasion by merozoites (“invadome”) [258] and iii) displayed a phenotype in *P. berghei* orthologue disruption that suggested an important role over the asexual blood stages. At the time of target selection (September-December 2015), data from PlasmoGEM was not yet publicly available. As a result, the rodent malaria phenotypes evaluated were genes deposited in the RMgmDB database. Of these targets, 14 (8.8%), 26 (16.4%) and 31 (19.5%) had a positive Tajima D score, were member of the “invadome” and had a rodent malaria phenotype reported (RMgmDB database), respectively.



**Figure 4.4: The number of targets predicted to have a signal peptide or transmembrane domains.** Figure showing proteins predicted to have a predicted N-terminal signal peptide and/or transmembrane domains (TMDs) in the A) 3D7 2D-immunoblots data set and B) 3D7 putative surface proteins.

First, I selected seven targets from the 14 with a positive Tajima D, primarily because their transcriptional profiles suggested an elevated transcriptional profile in

the late trophozoite and schizont stages of the life cycle (4 targets: 32-48 hpi, 2 targets 3-8 hpi). In addition, although they were under balancing selection [253] they had a lower median Tajima D score when compared to non-selected targets (N=7) (selected versus non-selected: 0.324 versus 0.7200, p-value=0.3829) representing less polymorphic candidates (Table 4.1). Second, 26 (16.4%) of the 159 targets have been proposed to be members of the invadome [258] (merozoite genes essential for erythrocyte invasion). I therefore selected ten under-studied targets as they may potentially make good immune targets.

Lastly, 31 (19.5%) of the 159 targets had rodent malaria orthologues in which genetic modification has been attempted (using data from the RMgmDB database). Some of the phenotypes reported either showed no differences in parasite growth rate and survival when compared to wild-type parasites. I therefore selected eleven targets whose attempts to genetically disrupt the *P. berghei* orthologues resulted in the following phenotypes; 1) had been attempted but were unsuccessful (N=4; PF3D7\_1455300, PF3D7\_1468100, PF3D7\_1025300 & PF3D7\_1105800). These may represent targets whose functions are essential and indispensable for parasite growth or survival or 2) targets whose phenotypes showed a reduced growth rate over the asexual blood stages of the lifecycle (N=2; Plasmeprin IV & Thioredoxin 2) or localised to the periphery of merozoites (N=2; *PfPAT* & *ISP3*), and therefore accessible to circulating antibodies. Three of the eleven selected targets encode the 6-cys (B9) antigen, a putative amino acid transporter and a tryptophan/threonine rich antigen. These were selected for the following reasons: The disruption of the *P. berghei* B9 protein resulted in arrested development of sporozoites within hepatocytes [433] and B9 contains the 6-cys domains which are thought to be involved in protein-protein interactions [407]. The identification of B9 in my proteomic studies, as well as in mass-spectrometry analysis of synchronous merozoites by Florens *et al* [254] may therefore represent a potential target of both pre-erythrocytic and blood-stage immunity. The second target, PF3D7-0629500, encodes a putative amino acid transporter, however its phenotype following disruption has not been reported yet. *P. falciparum* obtains the majority of its amino



acids from haemoglobin degradation, with the exception of isoleucine that is likely obtained from plasma via a transporter [471]. Blocking the function of this transporter through antibodies may therefore be detrimental for parasite survival, representing an attractive target. Lastly, PF3D7\_0830500 encoded a tryptophan-threonine rich antigen. The *P. vivax* members of the tryptophan-rich antigen family have been shown to be immunogenic [472] while one of *P. falciparum* orthologues of a protective *P. yoelii* antigen (pyAg-1) was shown to be a tryptophan rich antigen associated with the merozoite surface [473]. As expected, there was some overlap between selected targets as shown in Figure 4.2 and Table 4.1. For example, of the 10 invasion-related targets selected, 2 also had a phenotype described for their *P. berghei* orthologues. Similarly, two targets that had a positive Tajima D had a phenotype described in the RMgmDB database.

The last, five targets were selected as they were described as “conserved Plasmodium proteins, unknown function” whose maximum mRNA transcription was observed at the late schizont stage (N=1), merozoite stage (N=1) or was unknown (N=3). The selection of conserved proteins may be beneficial in downstream vaccine development, on the premise that protective immune responses elicited against these targets should be effective against diverse parasite strains from different geographical locations. This is of particular concern for *P. falciparum* vaccinology as the majority of the candidates are polymorphic and induce strain-specific responses [171, 222, 380]. A summary of the protein features and the available peer-reviewed literature of the 27 targets I selected for immunoprofiling are shown in Table 4.1. Included in this summary are phenotypes available on PlasmoGEM that are now publicly available.

**Table 4.1: A summary of the 27 antigens selected for immuno-profiling.**

Plasmodium falciparum gene ID	Product description	TMDs	Signal peptide	Timing of max. Transcription	Additional information on <i>P. berghei</i> genetic modification, role in erythrocyte invasion, evidence of balancing selection and studies on a naturally acquired immunity
PF3D7_0206200	Pantothenate transporter (PAT)	11	0	43	<i>Plasmodium falciparum</i> parasites are not viable over the asexual blood stages in the absence of pantothenic acid. <i>PfPAT</i> localises to the periphery of individual merozoites within schizonts. Genetic disruption of <i>PfPAT</i> was not successful and down-regulation of its expression resulted in parasite death <i>in vitro</i> [421]. Proposed to be essential for erythrocyte invasion[258]. <i>P. yoelii</i> gene disruption resulted in no mature oocysts or sporozoites[431]. In <i>P. berghei</i> gene disruption resulted in male and female gametocytes failing to egress from host erythrocytes[432].
PF3D7_0317100	6-cysteine protein (B9)	0	1	8	<i>P. berghei</i> gene disruption resulted in arrested development of sporozoites within hepatocytes[433](RMgmdB). Termed as dispensable on PlasmoGEM.
PF3D7_0525800	Inner membrane complex protein 1g, putative (IMC1g)	0	0	41	Proposed to be essential for erythrocyte invasion[258]. Termed as essential on PlasmoGEM.
PF3D7_0629500	Amino acid transporter, putative	9	0	45	<i>P. berghei</i> gene disruption was successful however the phenotype has not been described (RMgmdB).
PF3D7_0722200	Rhoptry-associated leucine zipper-like protein 1 (RALP1)	1	1	39	<i>P. falciparum</i> RALP1 has been refractory to gene knockout attempts[474] (RMgmdB) Proposed to be essential for erythrocyte invasion[258]. Shown to be recognised by a sera from asymptomatic adults in Thailand and Mali in one study[463] and China in a second study[263]. Termed as essential on PlasmoGEM.
PF3D7_0730800.2	Plasmodium exported protein, unknown function	0	1	48	
PF3D7_0822900	Conserved Plasmodium protein, unknown function	0	0	Unknown	Proposed to be essential for erythrocyte invasion[258].
PF3D7_0823800	DnaJ protein, putative	3	0	32	Evidence of balancing selection (Tajima D=0.324)[253]. Termed as resulting in a slow growth rate on PlasmoGEM following gene knockout.
PF3D7_0831400	Plasmodium exported protein, unknown function	1	1	Unknown	Evidence of balancing selection (Tajima D=0.132)[253]
PF3D7_0830500	Tryptophan/threonine-rich antigen (TryThrA)	1	0	3	<i>P. berghei</i> orthologues were successfully disrupted, however <i>P. falciparum</i> expresses multiple proteins encoded near subtelomeric regions therefore assigning orthology is not clear. Evidence of balancing selection (Tajima D=1.019)[253]
PF3D7_0925900	Conserved Plasmodium protein, unknown function	0	1	Unknown	

Plasmodium falciparum gene ID	Product description	TMDs	Signal peptide	Timing of max. Transcription	Additional information on <i>P. berghei</i> genetic modification, role in erythrocyte invasion, evidence of balancing selection and studies on a naturally acquired immunity
PF3D7_1014100	Conserved Plasmodium protein, unknown function	0	1	41	Proposed to be essential for erythrocyte invasion[258].
PF3D7_1025300	Conserved Plasmodium protein, unknown function	0	1	31	Three attempts to disrupt the gene were not successful (RMgMDB). Termed as essential on PlasmogEM.
PF3D7_1105800	Conserved Plasmodium protein, unknown function	1	1	39	Attempts to disrupt the gene were not successful (RMgMDB). Termed as essential on PlasmogEM.
PF3D7_1137300	Conserved Plasmodium membrane protein, unknown function	6	0	8	Evidence of balancing selection (Tajima D=1.015)[253]. Termed as resulting in a slow growth rate on PlasmogEM following gene knockout.
PF3D7_1229300	Conserved Plasmodium protein, unknown function	1	1	Unknown	Proposed to be essential for erythrocyte invasion[258].
PF3D7_1237900	Conserved Plasmodium protein, unknown function	1	1	Unknown	
PF3D7_1252300	Conserved Plasmodium protein, unknown function	1	0	Unknown	
PF3D7_1308000	Conserved Plasmodium membrane protein, unknown function	4	0	Unknown	Proposed to be essential for erythrocyte invasion[258].
PF3D7_1343700	Kelch protein K13 (K13)	0	0	47	Evidence of balancing selection (Tajima D=0.084)[253].
PF3D7_1345100	Thioredoxin 2 (TRX2)	0	1	43	<i>P. berghei</i> gene disruption showed a delayed blood-stage growth rate in mice[429]. Termed as resulting in a slow growth rate on PlasmogEM following gene knockout.
PF3D7_1401600	Plasmodium exported protein (PHISTb), unknown function	1	0	39	Proposed to be essential for erythrocyte invasion[258].
PF3D7_1407800	Plasmeprin IV (PM4)	1	0	48	<i>P. berghei</i> gene disruption showed a reduced growth rate and a virulence attenuated phenotype in mice[337] (RMgMDB). Evidence of balancing selection (Tajima D=0.656)[253]. Termed as resulting in a slow growth rate on PlasmogEM following gene knockout.
PF3D7_1455300	Conserved Plasmodium protein, unknown function	0	0	42	Two attempts to disrupt the <i>P. berghei</i> orthologues were not successful (RMgMDB) Termed as essential on PlasmogEM. Proposed to be essential for erythrocyte invasion[258]. Evidence of balancing selection (Tajima D=0.015)[253].
PF3D7_1460600	Inner membrane complex sub-compartment protein 3, putative (ISP3)	0	0	41	<i>P. berghei</i> GFP tagged parasites showed localisation to schizonts and merozoites[360]. Proposed to be essential for erythrocyte invasion[258].
PF3D7_1462300	Conserved Plasmodium protein, unknown function	3	1	1	
PF3D7_1468100	Conserved Plasmodium protein, unknown function	0	0	15	Two attempts to disrupt the <i>P. berghei</i> orthologues were not successful (RMgMDB) Termed as essential on PlasmogEM.

#### **4.5.1. Editing protein sequences for gene synthesis by GeneartAG**

The amino acid sequences for selected antigens were obtained from PlasmoDB and edited as described in section 4.4.2. The *P. falciparum* endogenous N-terminal signal peptides were replaced with the rat Cd4 domain 3 and 4 leader sequence had been shown to result in increased protein yields [469]. In addition, the predicted N-glycosylation sites were mutated to prevent addition of glycans to these sites. This is because the degree of N-linked glycosylation in native *P. falciparum* genes is controversial. Early studies on native immunoprecipitated MSP-1 and -2 proteins indicated that these antigens had no affinity for lectin nor were they sensitive to PNGase F treatment which suggested the absence of N-linked glycans [475]. Thereafter, bioinformatic analysis of the genome indicated that the parasite lacked most of the glycosyltransferases involved in this process [467] suggesting this glycosylation did not occur. However, three enzymes within this pathway are present, and it has been recently shown that *P. falciparum* synthesises a shorter version of N-glycans than those in mammalian cells [476, 477]. Lastly, the yield of recombinant protein increased following mutation of these glycosylation sites [469]. As a result, to avoid inappropriate addition of these glycans, the sites on the protein sequences were mutated prior to recombinant expression.

An example of the editing of a protein sequence (e.g. RALP1) is shown in Figure 4.5. The protein sequence for RALP1 (PF3D7\_0722200) is predicted to have a 22 amino acid N-terminal signal peptide and a transmembrane domain ranging between amino acids 3 and 20, as well as five predicted N-glycosylation sites. As shown in Figure 4.5, the protein sequence sent to GeneartAG excluded the predicted signal peptide and transmembrane domain (shown in red) and at predicted N-glycosylation sites (shown in yellow), the serine or threonine residues were replaced with alanine. The edited protein sequences that were submitted for codon-optimization and gene synthesis for each of the 27 selected antigens are available in Appendix 4.2.

**A.**

**>PF3D7\_0722200\_RALP1**

MNFFFFFFIIVYCFISSFYLIKSSNDKNKINKNNMPLQRGASNLKDINIHNVEKKSNMES  
VNNNINNTTCSKNLNANKVNPVNAHTGVNKKGETKNEEVEKEENENEEIEKEEIEENEENQQ  
NEEDENEENQQNEEDENEENQQMNVDVSAEKKNKELPEEKKELOKNSEKNINKEVIKD  
SNKDNTFNFHKNINTSFNKNKNIYNNNNNNNNNNKTFIEKKEEAIDMKKTHCSNNNMCK  
YNNMNNHMSTNKNNNQIFEEKQNVNKP IYNLKSLGNNTFKNDEKYNENDYKNNHDDDKK  
KYTDDSNEENIYEERNQKILLIHLKNIKELLNRQRQNFNNFLSFLSENYQSYEFYKSQ  
KYQNGKNYIDKLDQQGELKNVSVVTHSFLDMSKAANGKKDKNGV FVKLMNDQNDGDDTK  
DGDDTKDEDDHKNEDDHKNEDDHKNEDDHKNGDDNKGDDHKNGDDNKGDDDN  
GKKSHDISDIKNIIDTILQSDDI TDEQKKYLEI IKKILDLEEDVLNKEKEQLQLNKNIE  
VLMGKSELRNIAVNLKNGGDNESQRVDLAQNIVSNLLNFSVQLKNTGNIVYNNIQGQ  
GELLQSIIEKNIDKAENDLKKSTSVNTTFTPKNVPNTEQNDQETQTDDDINDSDIDENNT  
NDKNNCHMYKNNCHMYKNNCKKYFLNSHYNNYLCKKQKKYNKNDMGNKQKDNKKFKLN  
ESMKNHFYNI FVKETVMLKKLYKVLFEFLF

**B.**

**>PF3D7\_0722200\_RALP1 sent to GeneartAG**

SSNDKNKINKNNMPLQRGASNLKDINIHNVEKKSNMESVNNNINNTACSKNLNANKVNPV  
AHTGVNKKGETKNEEVEKEENENEEIEKEEIEENEENQQNEEDENEENQQNEEDENEENQQ  
MNVDVSAEKKNKELPEEKKELOKNSEKNINKEVIKDSNKDNTFNFHKNINTSFNKNKNI  
YNNNNNNNNNNKAFIEKKEEAIDMKKTHCSNNNMCKYNNMNNHMSTNKNNNQIFEEK  
QNVNKP IYNLKSLGNNTFKNDEKYNENDYKNNHDDDKKYTDDNEENIYEERNQKILLI  
LLKNIKELLNRQRQNFNNFLSFLSENYQSYEFYKSQKYQNGKNYIDKLDQQGELKNVAV  
VTHSFLDMSKAANGKKDKNGV FVKLMNDQNDGDDTKDGDDTKDEDDHKNEDDHKNEDDH  
KNEDDHKNEDDHKNGDDNKGDDHKNGDDNKGDDNGKKSHDISDIKNIIDTILQSDDI  
TDEQKKYLEI IKKILDLEEDVLNKEKEQLQLNKNIEVLMGKSELRNIAVNLKNGGDN  
EASQRVDLAQNIVSNLLNFAVQLKNTGNIVYNNIQGQGELLQSIIEKNIDKAENDLKKST  
SVNTTFTPKNVPNTEQNDQETQTDDDINDSDIDENNTNDKNNCHMYKNNCHMYKNNCK  
KYFLNSHYNNYLCKKQKKYNKNDMGNKQKDNKKFKLNE MKNHFYNI FVKETVMLKKLYK  
VLFEFLF

**Figure 4.5: Editing of protein sequences prior to GeneartAG gene synthesis.** Figure showing the protein sequence for RALP1 (A) in which the predicted N-terminal signal peptide (red) was excluded and N-glycosylation sites edited (yellow) (B).

#### **4.5.2. Successful sub-cloning of codon-optimised constructs into expression vector**

I received 26 out of the 27 GeneartAG constructs I requested for synthesis. For the 27<sup>th</sup> target, PF3D7\_0822900, 20 attempts to synthesise the gene by GeneartAG were unsuccessful. Synthesis problems often occur in sequences that either have repetitive sequences, have a high (>80%) or low (<20%) GC content or have long polypurine or polypyrimidine runs (<https://www.thermofisher.com/ke/en/home/life-science/cloning/gene-synthesis/geneart-gene-synthesis/geneart-standard-and-xxl-genes.html>). The PF3D7\_0822900 gene construct had several complexities that may

have contributed to the inability to synthesize it, including; 1) a window of 100 bases with a GC content of 19% and 2) multiple 8 base pair repeats were present between position 299 and 764. This construct was eliminated from subsequent study. For the 26 constructs I received, a summary of the expression pipeline and screening is summarised in Figure 4.6 and described in details below.

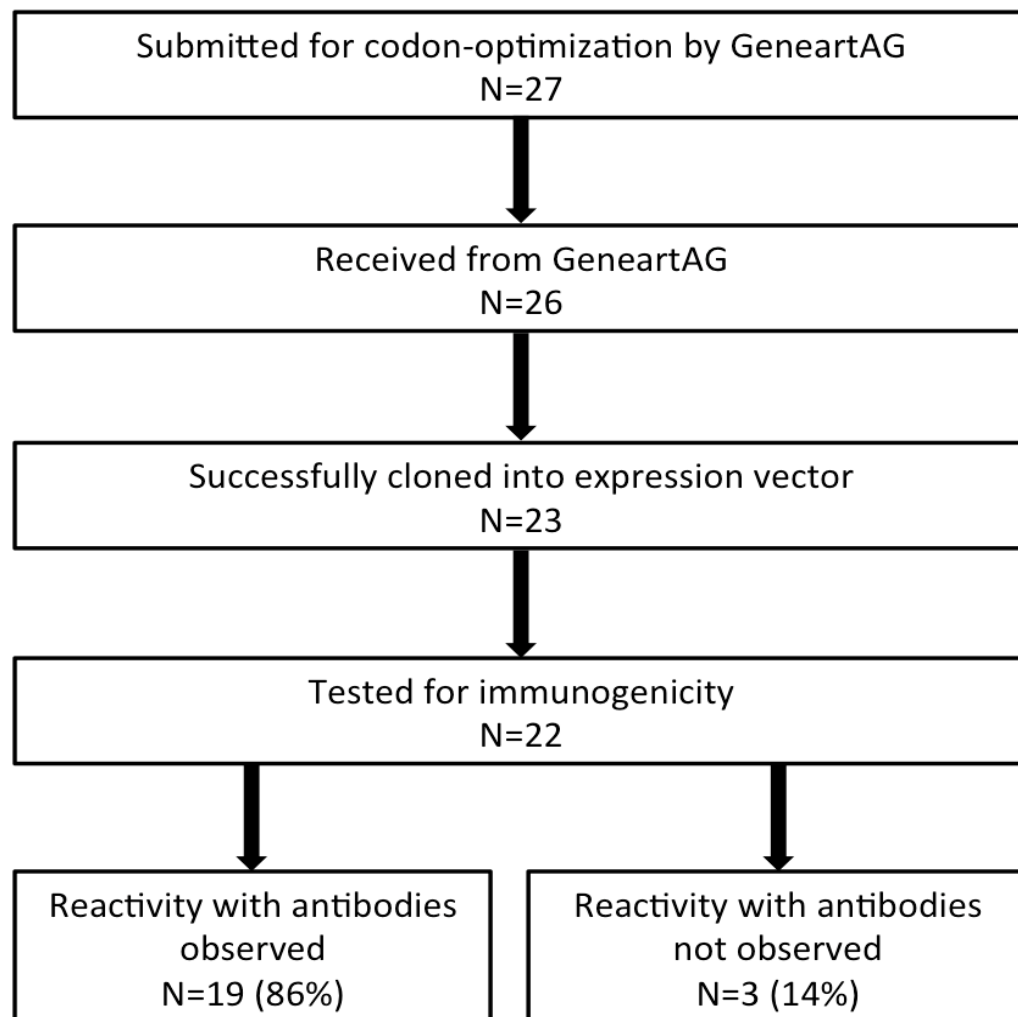
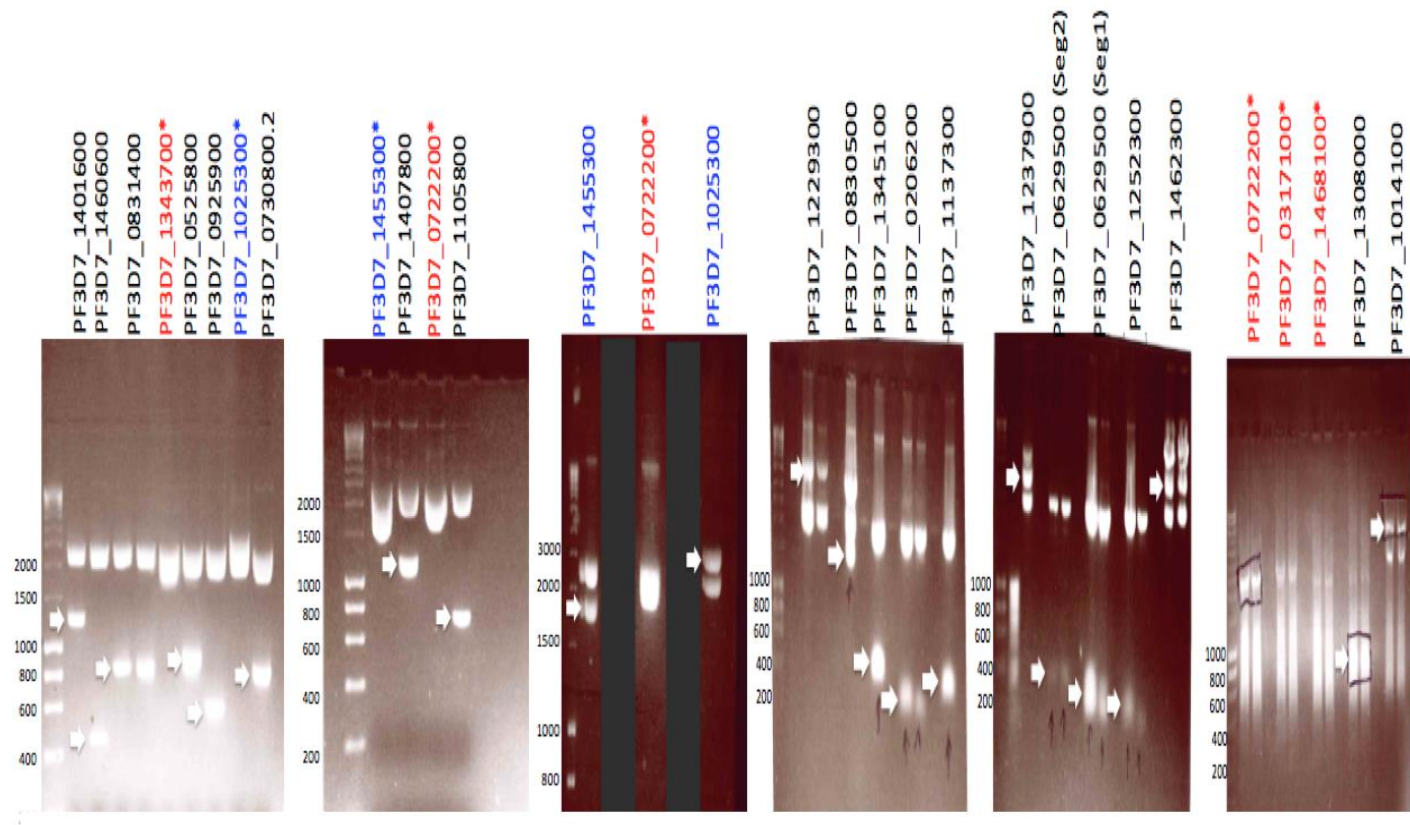


Figure 4.6: A summary of the selected and subsequently cloned and expressed antigens.

For the 26 constructs I received, the genes of interest (GOI) were first excised using restriction digestion using Not1 and Asc1, and separated on a 1% agarose gel. As shown in Figure 4.7, the expected GOI migrating at the *in silico* predicted size was obtained for 23 of the constructs. In the 23 targets in which restriction digestion was

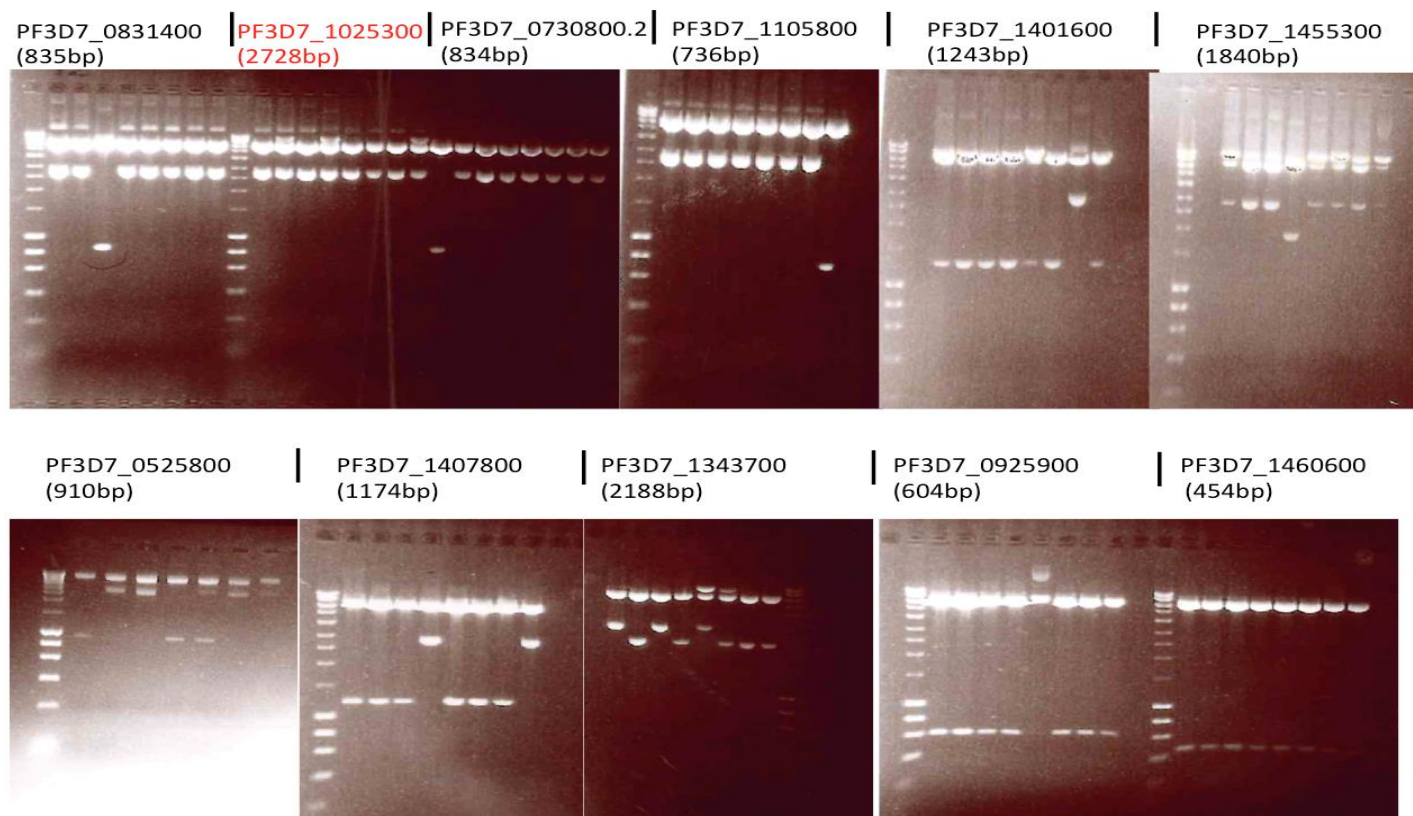
successful, I gel purified the GOI, ligated them to the expression vector and transformed *E.coli* cells with the ligation product. For each target, I screened 8 randomly selected colonies for the presence of my GOI. As shown in Figure 4.8, 4.9 and 4.10, a successfully ligated construct was observed in at least one of the 8 colonies screened in all 23 cases. Successfully cloned constructs were then expanded to obtain sufficient quantity of expression vector for transient transfection of expi293F cells. Figure 4.11 shows all the expression vectors for the 23 successful targets. These were used for subsequent recombinant protein expression.

Three of the twenty-six constructs received from GeneartAG were not successfully cloned to the expression vector. As shown in red in Figure 4.7, in the three targets, namely PF3D7\_0317100 (B9), PF3D7\_0722200 (RALP1) and PF3D7\_1468100, a clear separation of the GOI from backbone vector was not clear. In one target, PF3D7\_1343700 (KELCH), a clear separation was not observed however subsequent attempts were successful as shown in Figure 4.8 and 4.11. The genes encoding KELCH, RALP1 and B9 were expected to be 2188bp, 2857bp and 2191bp and migrated similarly to the backbone vector, which was 2400bp in size. The last GOI, PF3D7\_1468100 was expected migrate at 768bp. No product at this size was observed following restriction digestion. Due to the time constraints of my PhD, additional attempts to clearly separate the vector from the GOI have not yet been attempted. One approach to circumvent the problem would involve identification of additional restriction enzyme sites within the backbone vector downstream of the gene of interest. A triple digest should release the GOI at the expected size while cleaving the backbone vector into two fragments that would both be smaller in size and thus would separate more clearly from the GOI. An alternative approach would be to design vector-specific primers flanking my GOI, PCR amplify the GOI and restriction digest the PCR product and proceed with the ligation steps described above.

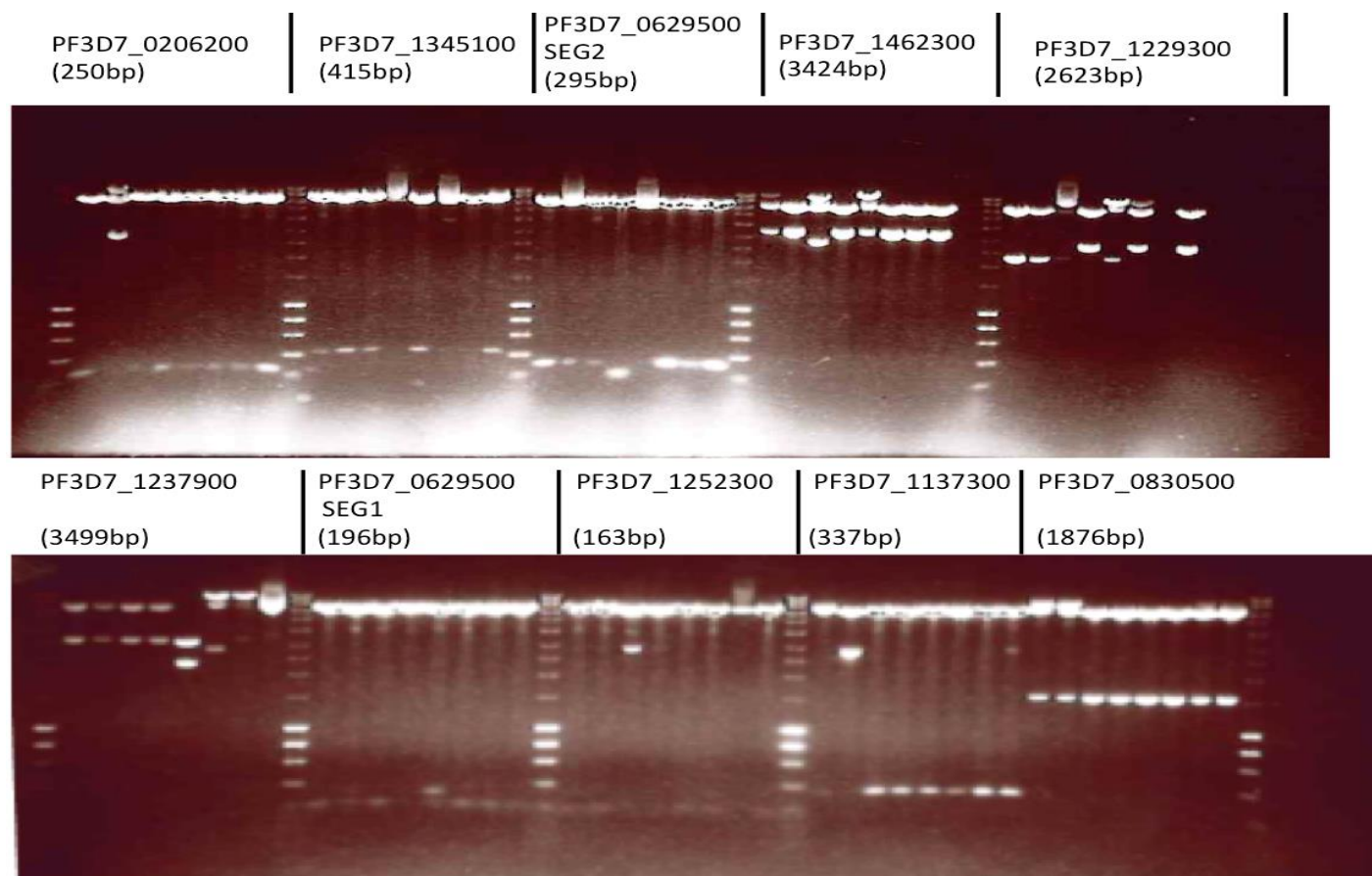


**Figure 4.7: Double digest of GeneartAG construct to release the GOI for subsequent sub cloning steps.** Figure showing a 1% agarose gel resolving the codon-optimised gene of interest excised from the backbone geneart vector. The GOI highlighted in red were not successfully separated from the geneart backbone vector as they migrated at similar sizes. White arrows shows the expected GOI following double digestion.

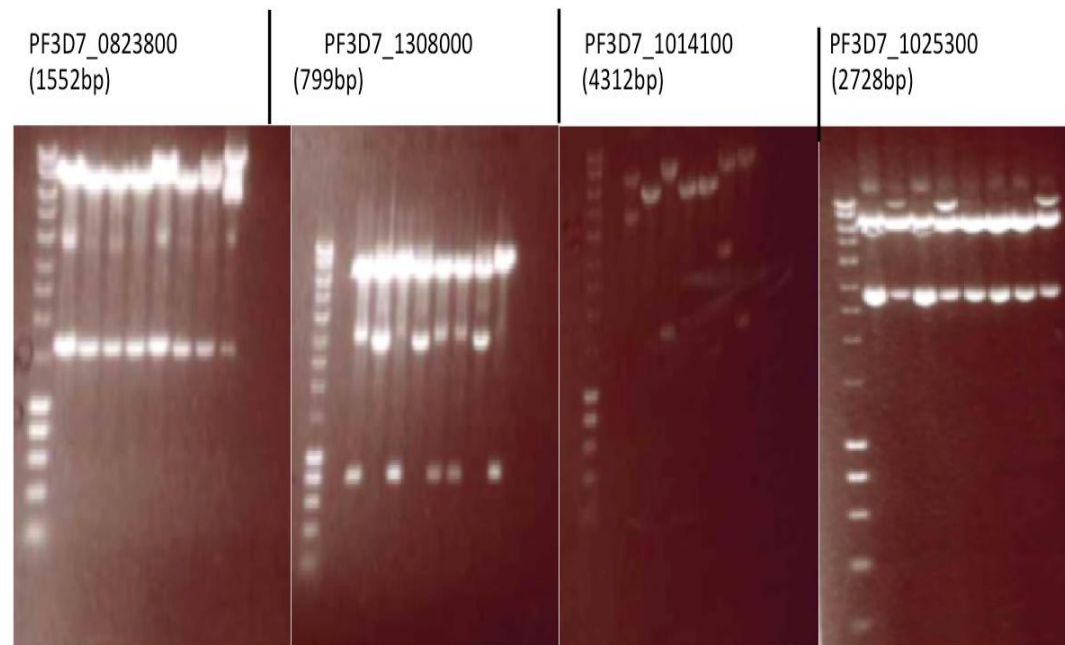




**Figure 4.8: *E.coli* colony screening to identify successfully ligated expression vector.** Screening 8 colonies per GOI for successful sub-cloning of target to each expression vector. Target labelled in red shows a colony screen that was not successful. In the remainder, a GOI at the expected base pair size (shown in brackets) was evident in at least one of the eight colonies.



**Figure 4.9: *E.coli* colony screening to identify successfully ligated expression vector.** Screening 8 colonies per GOI for successful sub-cloning of target to each expression vector. The GOI at the expected base pair size (shown in brackets) was evident in at least one of the eight colonies.



**Figure 4.10: *E.coli* colony screening to identify successfully ligated expression vector.** Screening 8 colonies per GOI for successful sub-cloning of target to each expression vector. In the remainder, a GOI at the expected base pair size (shown in brackets) was evident in at least one of the eight colonies.

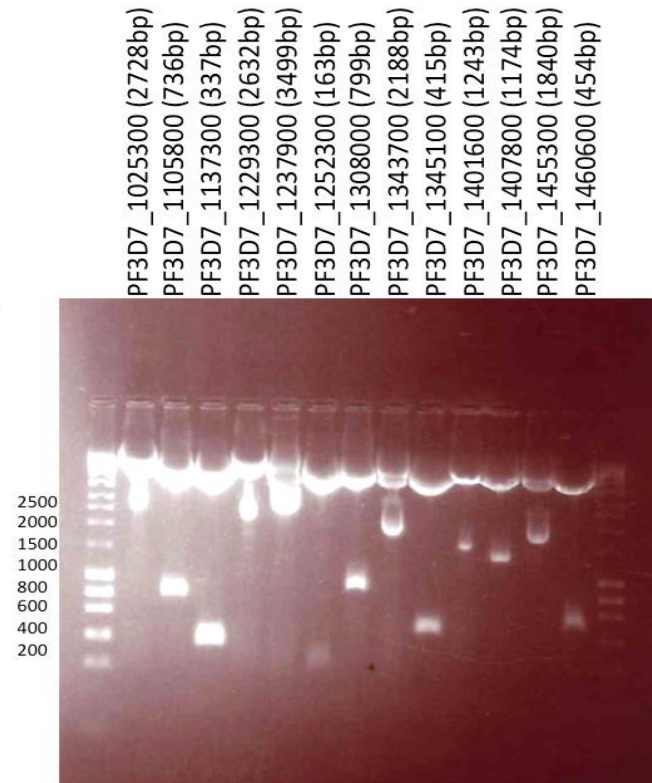
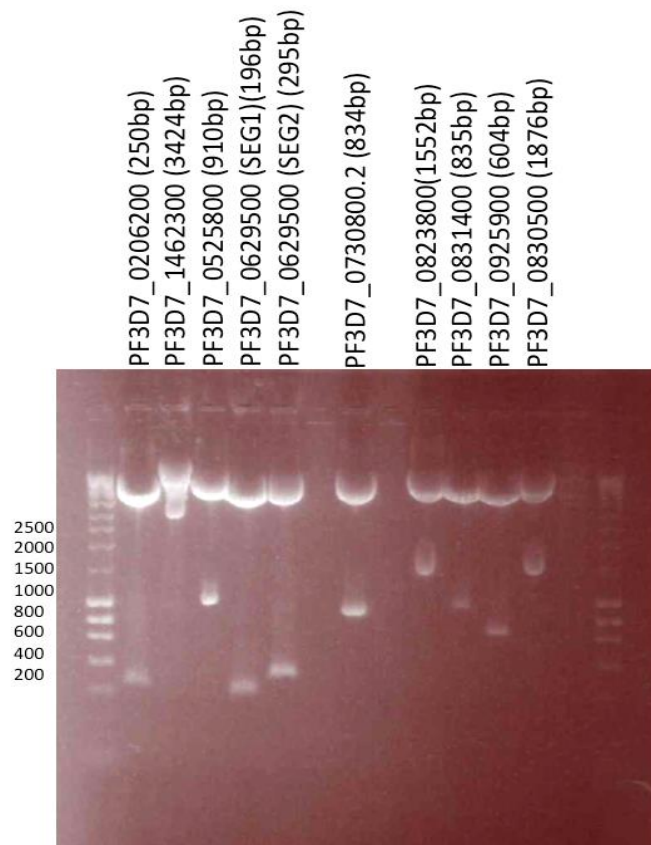
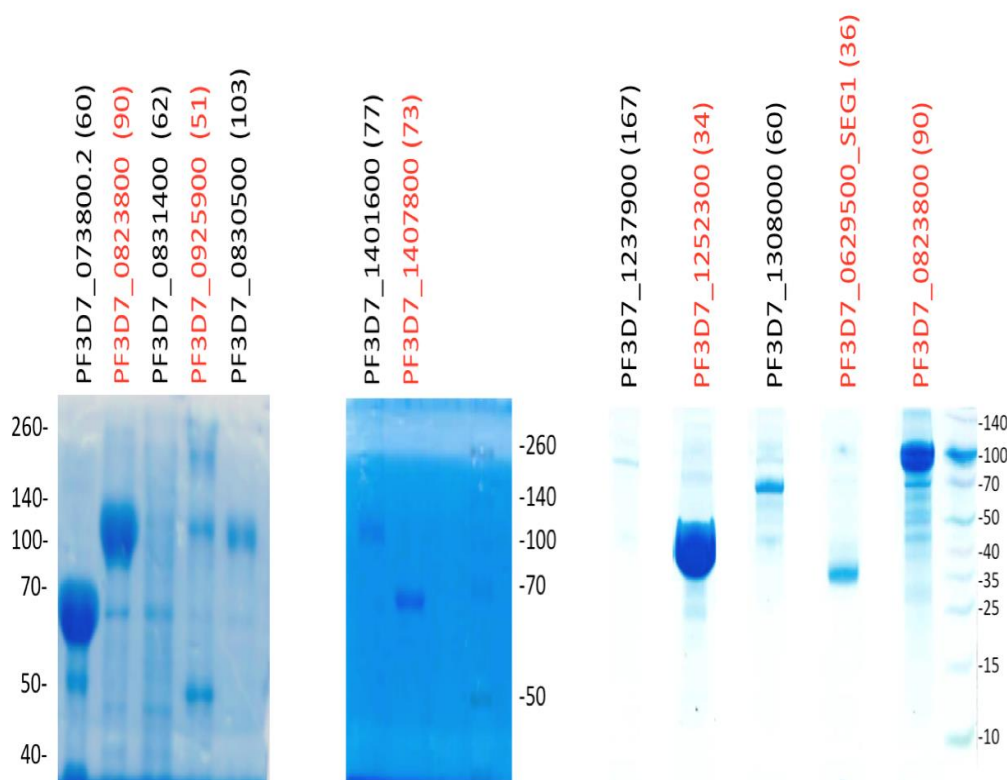


Figure 4.11: A summary of successfully cloned geneart constructs to the expression vector

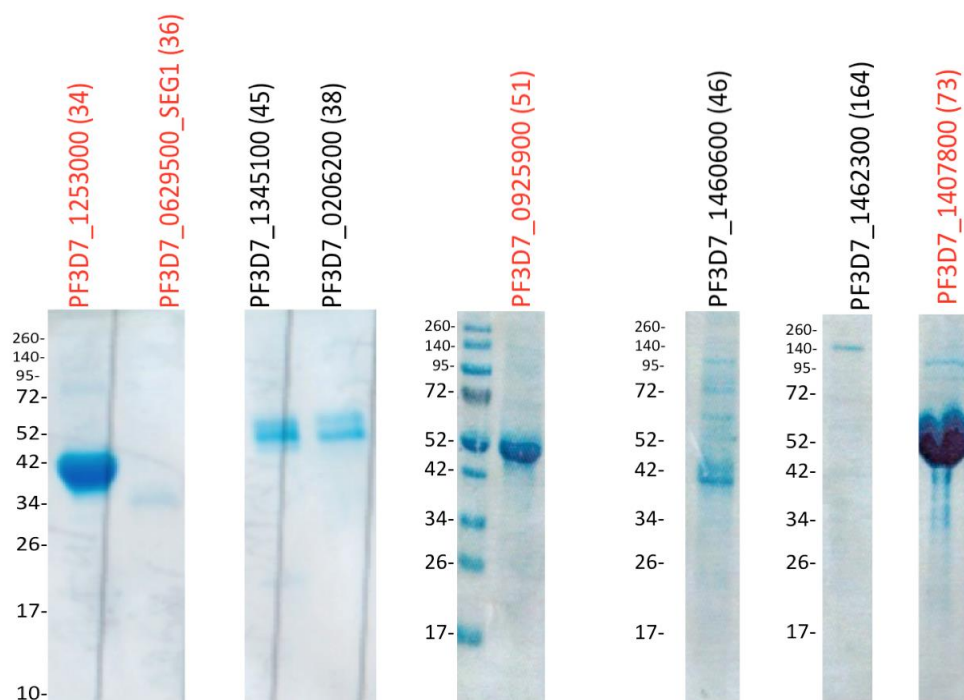
### 4.5.3. Recombinant protein expression

To obtain recombinant proteins, I transfected expi293F cells with the successfully sub-cloned Geneart constructs as shown in Figure 4.11. The recommended starting cell cultures for transfection were 30ml at a cell density of  $2.0 \times 10^6$  cells/ml (total cell density= $6.0 \times 10^7$  cells), and 30 $\mu$ g of expression vector is recommended. For each of the 22 targets, varying concentration of protein was obtained ranging from 150 $\mu$ g/ml to 1490 $\mu$ g/ml. The expi293F expression system is scalable allowing transfection of upto  $1.0 \times 10^9$  cells, meaning that sufficient quantities of protein are able to be generated for all targets. One target, PF3D7\_1014100 was not used for transfection, as sufficient quantity of DNA for transfection had not been obtained. As shown in Figure 4.12, 4.13 and Table 4.2, purified recombinant proteins were obtained and migrated at the expected predicted molecular weights when separated on a reducing SDS-page gel following purification using the Ni-NTA system (Invitrogen).



**Figure 4.12: Coomassie stained SDS gels showing recombinant proteins.** Three Coomassie stained SDS gels showing eleven purified recombinant proteins. The numbers in bracket indicate the predicted molecular masses (in KDa) for each protein. Protein in red indicates the same target expressed and purified in two independent experiments.





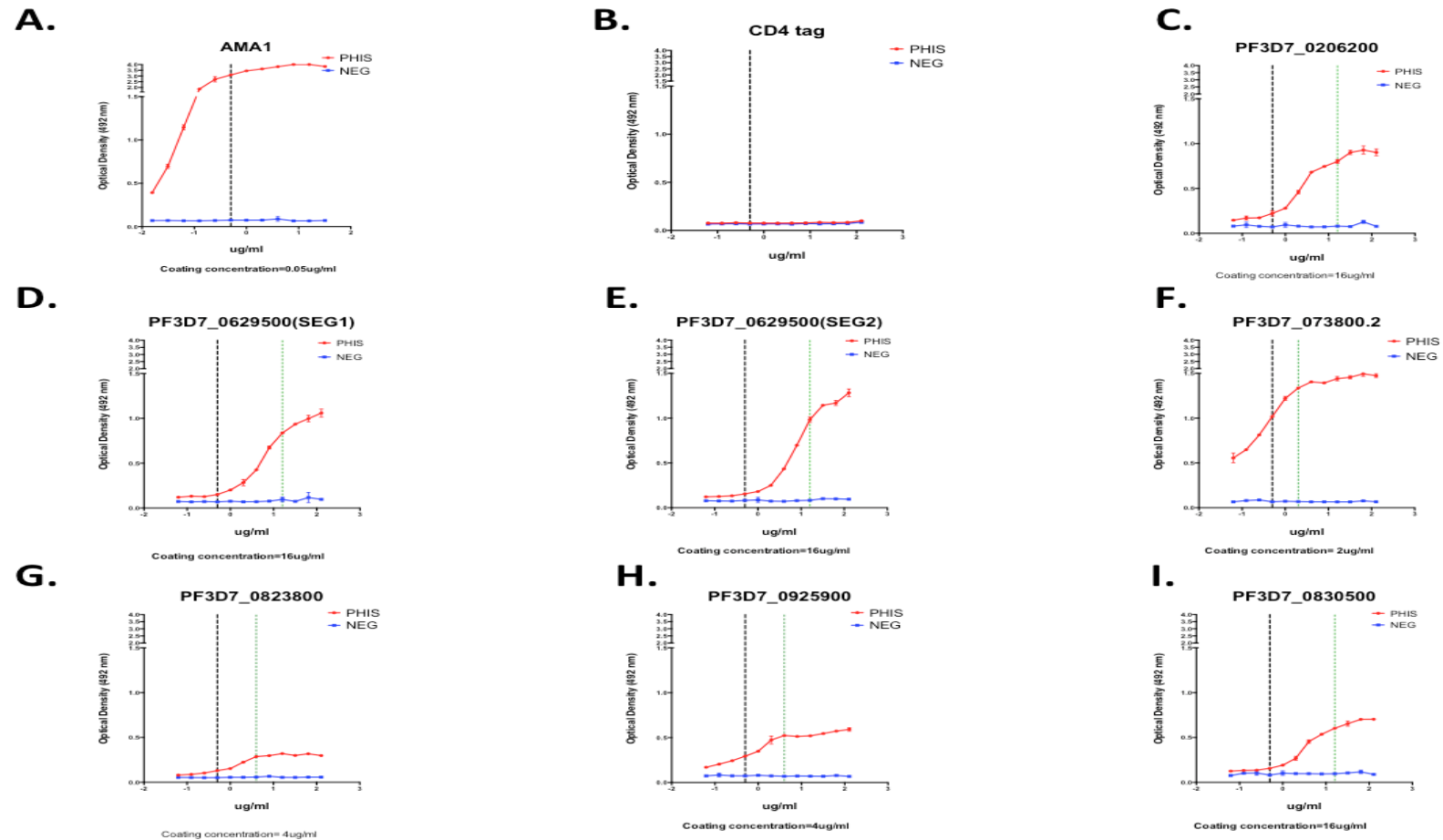
**Figure 4.13: Coomassie stained SDS gels showing recombinant proteins.** Two Coomassie stained SDS gels showing four additional purified recombinant proteins. The numbers in bracket indicate the predicted molecular masses (in KDa) for each protein. Protein in red indicates the same target expressed and purified in two independent experiments.

#### 4.5.4. Identifying reactogenic targets using ELISA

A total of 27 antigens were selected for immunoprofiling and 22 targets were tested for immunogenicity using a pool of hyper immune sera (PHIS) from adults living in the malaria endemic region, Kilifi, Kenya. A pool of sera from adults living in Sweden who have not been exposed to malaria was used as a negative control. For the 22 antigens for which recombinant protein was obtained, reactogenicity was measured by measuring a two-fold serial dilution of recombinant antigen at a fixed dilution (1:1000) of a positive and negative serum samples. As shown in Figure 4.14, 4.15 and 4.16, 19 (86%) of the selected targets showed a dose response curve with the positive control sera. No response to these antigens was observed with the Swedish non-exposed sera even at the highest protein concentration tested. Sixteen (73%) of selected and expressed antigens displayed a dose response curve that reached saturation as shown in Figure 4.14, 4.15 and 4.16A. AMA1 recombinantly expressed using the same system was included as a comparator. As shown in Figure 4.14A, AMA1 titres with the positive control reached the highest optical density measurements

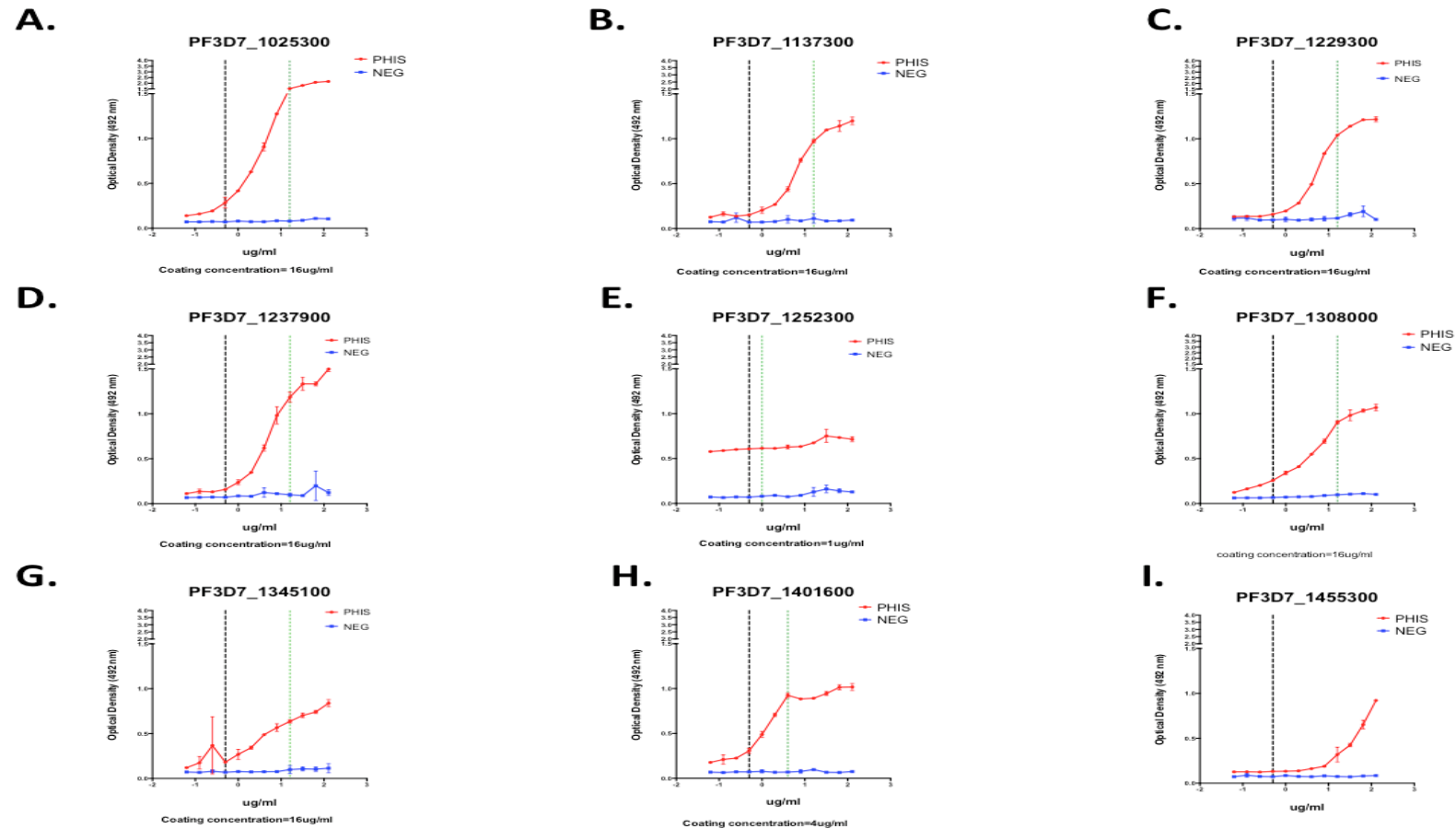
observable (OD=4). The selected targets were less reactogenic than AMA1, with none of them attaining an optical density of 4. However, AMA1 is known to be a highly immunodominant antigen and the differences do not necessarily reflect on the vaccine potential of the new targets.

A second approach, in which the optimal ELISA well coating concentration was determined for each antigen (shown as a green line in Figure 4.14, 4.15 and 4.16) also indicated that these targets were less immunogenic compared to the AMA1. In all examples, the coating concentration was over 2 fold above the 0.05µg/ml concentration determined for AMA1. In Figure 4.16 (D, F and G), 3 of the 22 antigens that were immunogenic showed evidence of a dose response curve however this did not reach saturation. Three antigens (14%) showed no evidence of immunogenicity: these were PF3D7\_0525800 (IMC1g), PF3D7\_0831400 and PF3D7\_1343700 (KELCH13) (Figure 4.16 B, C and E). As expected, the CD4 tag present in each antigen was not immunogenic as shown in Figure 4.14B. Table 4.2 provides a summary of the selected proteins and indicates the immunogenic, non-immunogenic and targets that were not tested because they dropped out at different stages in the synthesis, cloning and expression pipeline.

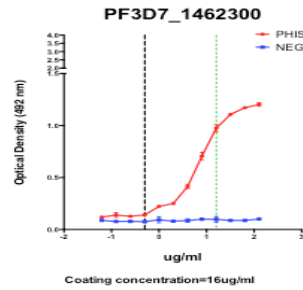
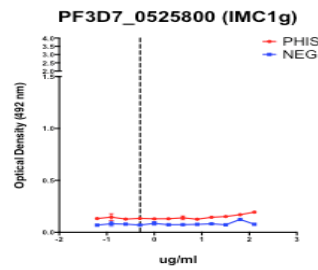
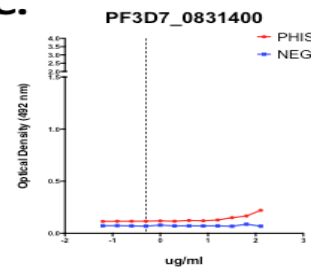
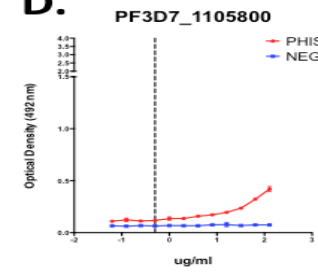
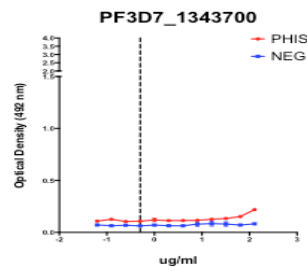
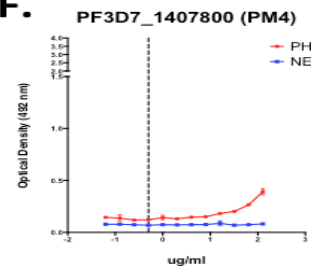
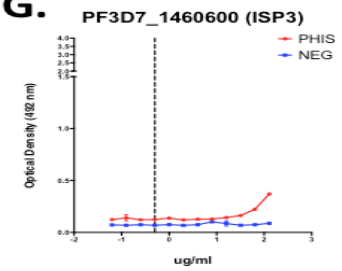


**Figure 4.14: Immunogenicity of recombinant antigens measured by ELISA.** Figure showing the immunogenicity of 7 of the 22 selected recombinant proteins. For comparison AMA1 (A) was recombinantly expressed and assayed similarly to the novel antigens. The CD4 tag present on each antigen was not immunogenic (B). The black line shows the coating concentration selected for AMA1 while the green line indicates the coating concentration for each antigen.





**Figure 4.15: Immunogenicity of recombinant antigens measured by ELISA.** Figure showing the immunogenicity of the 9 of the 22 selected recombinant proteins. The black line shows the coating concentration selected for AMA1 while the green line indicates the coating concentration for each antigen.

**A.****B.****C.****D.****E.****F.****G.**

**Figure 4.16: Immunogenicity of recombinant antigens measured by ELISA.** Figure showing the immunogenicity of the 7 of the 22 selected recombinant proteins. The black line shows the coating concentration selected for AMA1 while the green line indicates the coating concentration for each antigen.

**Table 4.2: Table showing novel immunogenic targets identified from screening by ELISA and the sections of the protein evaluated.**

Plasmodium falciparum gene ID	Region expressed (Number of amino acids)	Predicted molecular weight*	Product description	Additional information on <i>P. berghei</i> genetic modification, role in erythrocyte invasion, evidence of balancing selection & studies on a naturally acquired immunity
PF3D7_0206200	D241-K319 (79)	38	Pantothenate transporter (PAT)	<i>Plasmodium falciparum</i> parasites are not viable over the asexual blood stages in the absence of pantothenic acid. PfPAT localises to the periphery of individual merozoites within schizonts. Genetic disruption of PfPAT was not successful and down-regulation of its expression resulted in parasite death <i>in vitro</i> [421]. Proposed to be essential for erythrocyte invasion[258]. <i>P. yoelii</i> gene disruption resulted in no mature oocysts or sporozoites[431]. In <i>P. berghei</i> gene disruption resulted in male and female gametocytes failing to egress from host erythrocytes[432].
PF3D7_0525800	C2-A300 (299)	63	Inner membrane complex protein 1g, putative (IMC1g)	Proposed to be essential for erythrocyte invasion[258].
PF3D7_0629500#	Q331-R391: G451-N544 (61:94)	36,40	Amino acid transporter, putative	<i>P. berghei</i> gene disruption was successful however the phenotype has not been described.
PF3D7_0730800.2	S20-F293 (274)	60	Plasmodium exported protein, unknown function	
PF3D7_0823800	Y93-K605 (513)	90	DnaJ protein, putative	Evidence of balancing selection (Tajima D=0.324)[253].
PF3D7_0831400	K29-I302 (274)	62	Plasmodium exported protein, unknown function	Evidence of balancing selection (Tajima D=0.132)[253]
PF3D7_0830500	K55-V675 (621)	103	Tryptophan/threonine-rich antigen (TryThrA)	<i>P. berghei</i> orthologues were successfully disrupted, however <i>P. falciparum</i> expresses multiple proteins encoded near subtelomeric regions therefore assigning orthology is not clear. Evidence of balancing selection (Tajima D=1.019)[253]
PF3D7_0925900	S21-D217 (197)	51	Conserved Plasmodium protein, unknown function	
PF3D7_1025300	Y18-F922 (908)	135	Conserved Plasmodium protein, unknown function	Three attempts to disrupt the gene were not successful

Plasmodium falciparum gene ID			Product description	Additional information on <i>P. berghei</i> genetic modification, proposed to be essential for erythrocyte invasion, evidence of balancing selection, studies on a naturally acquired immunity
PF3D7_1105800	D26-Y266 (241)	57	Conserved Plasmodium protein, unknown function	Attempts to disrupt the gene were not successful
PF3D7_1137300	L582-D689 (108)	42	Conserved Plasmodium membrane protein, unknown function	Evidence of balancing selection (Tajima D=1.015)[253].
PF3D7_1229300	K21-S890 (870)	132	Conserved Plasmodium protein, unknown function	Proposed to be essential for erythrocyte invasion[258].
PF3D7_1237900	E27-S1188 (1162)	167	Conserved Plasmodium protein, unknown function	
PF3D7_1252300	S2-L51 (50)	34	Conserved Plasmodium protein, unknown function	
PF3D7_1308000	P341-K602 (262)	60	Conserved Plasmodium membrane protein, unknown function	Proposed to be essential for erythrocyte invasion[258].
PF3D7_1343700	E2-I726 (725)	112	Kelch protein K13 (K13)	Evidence of balancing selection (Tajima D=0.084)[253].
PF3D7_1345100	T24-L157 (134)	45	Thioredoxin 2 (TRX2)	<i>P. berghei</i> gene disruption showed a delayed blood-stage growth rate in mice[429].
PF3D7_1401600	K69-I478 (410)	77	Plasmodium exported protein (PHISTb), unknown function	Proposed to be essential for erythrocyte invasion [258].
PF3D7_1407800	S63-L449 (387)	73	Plasmepsin IV (PM4)	<i>P. berghei</i> gene disruption showed a reduced growth rate and a virulence attenuated phenotype in mice[337]. Evidence of balancing selection (Tajima D=0.656)[253].
PF3D7_1455300	D2-R609 (608)	98	Conserved Plasmodium protein, unknown function	Two attempts to disrupt the <i>P. berghei</i> orthologues were not successful Proposed to be essential for erythrocyte invasion[258]. Evidence of balancing selection (Tajima D=0.015)[253].
PF3D7_1460600	G2-A148 (147)	46	Inner membrane complex sub-compartment protein 3, putative (ISP3)	<i>P. berghei</i> GFP tagged parasites showed localisation to schizonts and merozoites[360]. Proposed to be essential for erythrocyte invasion[258].

Plasmodium falciparum gene ID			Product description	Additional information on <i>P. berghei</i> genetic modification, proposed to be essential for erythrocyte invasion, evidence of balancing selection, studies on a naturally acquired immunity
PF3D7_1462300	I19-F1155 (1137)	164	Conserved Plasmodium protein, unknown function	
Antigens in which recombinant forms were not obtained				
PF3D7_0317100	G22-F969 (948)	141	6-cysteine protein (B9)	<i>P. berghei</i> gene disruption resulted in arrested development of sporozoites within hepatocytes[433].
PF3D7_0722200	S23-F749 (726)	114	Rhoptry-associated leucine zipper-like protein 1 (RALP1)	<i>P. falciparum</i> RALP1 has been refractory to gene knockout attempts[474]. Proposed to be essential for erythrocyte invasion[258]. Shown to be recognised by a sera from asymptomatic adults in Thailand and Mali in one study[463] and China in a second study[263].
PF3D7_0822900 <sup>s</sup>	E2-K1176 (1175)	<sup>s</sup>	Conserved Plasmodium protein, unknown function	Proposed to be essential for erythrocyte invasion[258].
PF3D7_1014100	E22-P1455 (1433)	200	Conserved Plasmodium protein, unknown function	Proposed to be essential for erythrocyte invasion[258].
PF3D7_1468100	S2-I2558 (2557)	324	Conserved Plasmodium protein, unknown function	Two attempts to disrupt the <i>P. berghei</i> orthologues were not successful

Key: \*- Predicted molecular weight in kilodaltons including the CD4-hexahistidine tag on the C-terminal end of protein. #- Two extracellular loops were predicted and expressed as segment 1 and 2. \$-Geneart construct was not available.

Blue: immunogenic and achieved saturation. Purple: evidence of immunogenicity. Orange: not immunogenic. White- not tested.

## 4.6. Discussion

### *Nineteen novel potential vaccine candidates identified*

I have identified a set of 19 novel antigens as targets of naturally acquired antibody responses and therefore potential vaccine candidates. These included six targets proposed to play essential roles in merozoite invasion of erythrocytes [258], four targets considered to be under balancing selection [253] but had not previously been evaluated for naturally acquired antibody responses and five targets whose functions are unknown. These represent a pool of ideal targets for pre-clinical evaluation as potential blood-stage vaccine candidates. Within this set, particularly interesting targets that warrant further evaluation include the conserved targets that have been proposed to be essential for invasion of erythrocytes such as the three antigens, PF3D7\_1229300, PF3D7\_1308000 and PF3D7\_1455300. Identifying conserved targets is important because they may overcome the hurdle of parasite genetic diversity that has contributed to the low efficacy of previous vaccine candidates such as RTS'S [171], AMA1 [172] and MSP-2 [222]. Additional targets of interest include the transporters *PfPAT* and a putative amino acid transporter that have been shown to be immunogenic in this study for the first time. Not only has *PfPAT* been shown to localise to the periphery of individual merozoites [421], attempts to disrupt the *P. falciparum* gene were unsuccessful [421] and it has been proposed to be essential for invasion [258] suggesting it may play an indispensable role in parasite biology. Similarly, blocking the active in-take of essential amino acids such as isoleucine may prove detrimental to *P. falciparum* warranting further evaluation of the amino-acid transporter as a target of protective antibodies.

### *Stringent selection criteria results in a high immunogenicity hit rate*

Encouragingly, 86% of the selected antigens showed evidence of immunogenicity. This hit rate was higher than has been observed in studies that solely mined PlasmoDB for potential blood-stage immunogenic antigens. For example, in a study that selected 138 antigens prioritized on the presence of a signal sequence and/or transmembrane domains and elevated transcription profile in the schizont stages,

only 21.7% of them were found to be immunogenic [263]. Similarly, rapid *in vitro* transcription/translation expression systems have allowed the large-scale recombinant expression of *P. falciparum* open reading frames [265, 269]. However, the hit rate on immunogenic targets in these studies is low. For instance, in one study, 2320 *P. falciparum* protein fragments were screened by protein arrays using sera from Malian children and adults. Only 491 (21%) targets were found to be immunogenic [265]. In a second study, an analysis of 824 *P. falciparum* proteins identified 163 (20%) targets as being immunogenic when measured using sera from Kenyan immune adults [268]. The higher hit rate in my study can be attributed to focusing on proteins obtained from merozoites, and identified following reactivity with a pool of hyper-immune sera and likely to be located on the surface of the merozoite. In addition, it has been recognised that RNA levels may not always correlate to protein levels due to different rates of mRNA translation and/or protein degradation that influences a proteins half-life [478, 479]. Therefore focusing on proteomic data as opposed to transcription levels ensured the presence of target protein during blood-stages of *P. falciparum* infection. In addition, employing stringent down selection criteria increased the likelihood of identifying true targets of naturally acquired antibodies by eliminating false positives hits common to proteomic analyses.

#### ***Comparative immuno-reactivity of novel proteins with known blood-stage vaccine candidate***

The merozoite antigen AMA1 is a well-studied immunogenic blood-stage antigen that demonstrated low efficacy (less than 10%) in a phase 2 trial involving children in Mali over a 2-year follow-up period[172], largely due to the fact that strain-specific antibodies appear to dominate anti-AMA1 responses. The full-length ectodomain of AMA1 expressed at similar levels to the novel antigens, and was used as a comparator to determine the relative immunogenicity of newly identified targets. In general, the nineteen targets identified in these analyses were less immunogenic than AMA1, evidenced by the lower optical density observed at saturation and the higher coating concentration required to observe antibody

reactivity. These findings were expected, as the majority of them are conserved targets and only 7 (32%) of selected targets had evidence of balancing selection as indicated by a positive Tajima D [253]. Highly polymorphic targets often result from being under strong immune pressure and are evident in immunodominant targets[253]. Furthermore, these scores were on average lower than the scores for AMA1 and other known immunogenic merozoite targets. Nevertheless, two targets namely PF3D7\_073800.2 (*Plasmodium* exported protein, unknown function) and PF3D7\_1025300 (conserved *Plasmodium* protein, unknown function) achieved relatively high optical density values on saturation when compared to the other novel targets.

Three targets showed no evidence of antibody reactivity. These included PF3D7\_0525800 (IMC1g), a putative member of the inner membrane complex (IMC). As the IMC is located beneath the plasma membrane, it is plausible that this target is not exposed to circulating antibodies. The other targets that were not immunogenic included PF3D7\_0831400 and PF3D7\_1343700 whose functions have not been elucidated. Interestingly, while these targets had a positive Tajima D score, the score was low, 0.132 and 0.084 respectively[253]. RALP1 was the only target selected in my study that has been previously evaluated in the context of naturally acquired immunity using sera from asymptomatic adults in Thailand[463], Mali[463] and China[263]. However, I was unable to express this target, meaning I could not compare my data with available literature.

### ***Conserved targets are potential ideal vaccine candidates***

Fifteen (79%) of the reactogenic novel targets examined in this study were identified as conserved or of limited polymorphism (and therefore not under balancing selection) in a genome-wide population genetic analysis of Gambian isolates[253]. In addition, the majority of these targets are described as conserved proteins of unknown function on PlasmoDB, representing ideal targets that may overcome one of the major obstacles hindering vaccine development. Parasite antigenic diversity has contributed to the low effectiveness observed in the majority



of multi-subunit vaccine candidates tested in phase 2 and 3 trials[380, 480]. Clinical trials using the following candidates, RTS'S (CSP)[171], combination B (MSP-1, -2 and RESA)[373] and AMA1[172] have been shown to induce strain-specific responses and ultimately resulted in low vaccine efficacies against heterologous strains. Multivalent (vaccine consisting of multiple alleles of the same antigen) or a multi-subunit (vaccine consisting of multiple distinct antigens) approaches may be better and are under evaluation. An alternative approach would be to target a relatively conserved antigen with the expectation that vaccine induced responses would be effective across diverse parasite isolates. This has been demonstrated for the conserved target reticulocyte binding protein homologue 5 (RH5), in which only 5 non-synonymous SNPs have been reported at over a 10% frequency in 290 clinical isolates from diverse geographical locations[454]. Antibodies raised against the Pf3D7 variant of RH5 showed broad inhibitory activity against diverse *P. falciparum* lab isolates and strains[454, 455] in growth inhibition *in vitro* assays. In subsequent vaccination followed by challenge studies, *Aotus* monkeys vaccinated with the Pf3D7 variant of RH5 and challenged with the virulent heterologous FVO strain showed reduced parasitaemia and ability to self-cure when compared to control animals[456]. This demonstrated the *in vivo* vaccine induction of responses that were protective against heterologous strains, showing the utility of using a conserved vaccine candidate. This similar protection against homologous and heterologous *P. falciparum* strains has yet to be demonstrated in human clinical trials. Nevertheless, a pool of conserved blood-stage targets of naturally acquired antibody responses warrants further pre-clinical evaluation as vaccine candidates.

### ***Recombinant expression of functionally relevant antigens***

I successfully obtained soluble recombinant full-length extracellular domains that ranged from 34-167 kDa in size (Table 4.2), for 16/26 proteins that either lacked or had a single transmembrane domain. In an additional six antigens that were multi-transmembrane proteins: the longest predicted extracellular loops were similarly obtained in soluble form. This resulted in an 84% success rate in the heterologous expression of soluble *P. falciparum* proteins which was similar to the rates observed

in studies that expressed *P. falciparum* [469] and *P. vivax* [481] merozoites proteins. This were considerably higher than efforts using the *E.coli* system by Mehlin *et al* [482], Aguiar *et al* [483] and Vedadi *et al* [484], that obtained soluble proteins for 6.3% and 7.3% and 20.9% of selected targets respectively. My success rate is attributed to several factors: I selected a mammalian expression system using commercially available Exp293F cells and codon-optimised my gene constructs to facilitate expression in human cells. In addition, I mutated the N-glycosylation sites preventing inappropriate addition of N-glycans and replaced the native *P. falciparum* signal peptide with an exogenous peptide that drives mouse antibody secretion. These modifications had been shown to increase the protein yield obtained using HEK293E cells[468, 469]. The mammalian expression system was selected over bacterial or the cell-free systems available within our laboratory for the following reasons. Although bacterial expression systems are relatively easy to handle and cost-effective, soluble proteins are seldom obtained [482-484] and they are limited to proteins less than 50 kDa[485] (74% of selected targets were >50 kDa; Table 4.2). This has subsequently resulted in expression of fragments of proteins by *E.coli* as opposed to full-length proteins that can be obtained with mammalian cells [468]. The cell-free systems such as the eukaryotic wheat germ cell-free protein synthesis system (WGCFs)[269] have been used successfully to obtain soluble *P. falciparum* proteins [269]. However, an inverse correlation was also observed between soluble proteins and size [269] resulting in the expression of segments of proteins separately [265, 269, 271, 486]. *Plasmodium* proteins >200 kDa in size have been successfully expressed in mammalian cells, and have recapitulated known biochemical activity such as the MSP1-MSP7 and P12-P41 protein-protein interactions[469]. Although the yields using mammalian systems are lower when compared to *E.coli* expression systems, the system is scalable and antibody detection assays have improved from the ELISA to luminex and protein arrays that require over 100-fold less antigen for detection in cohort studies.

## ***Conclusion***

In summary, I identified 19 novel antigens that were reactogenic with a pool of hyper-immune sera from adults naturally exposed to *P. falciparum* infection. The majority of these targets are of unknown function, of limited polymorphism and may play essential roles in merozoite invasion of erythrocytes. They represent ideal candidates for pre-clinical evaluation as potential vaccine candidates. These would include evaluating the prevalence and titres of antibody responses to these targets in individuals living in malaria endemic areas and their correlation with immunity or susceptibility to clinical episodes of *P. falciparum* malaria. In addition, identifying the protective mechanisms by which antibodies to each individual target function and the extent to which this function is strain transcending would provide valuable information for vaccine target prioritization. In line with this, a subset of the nineteen antigens has been evaluated using sera from a longitudinally monitored cohort of immune adults. In chapter 5, I present data on associations between antibody responses and clinical immunity. In chapter 6, I present data on the correlation between antibody responses to novel targets and protective antibody effector functions.

## **CHAPTER 5**

### **Antibodies against novel targets in a longitudinally monitored cohort of adults**

#### **5.0. Introduction**

Adults living in an area with a high intensity of malaria transmission are known to be immune to clinical episodes of malaria even in the presence of detectable parasites [11, 76], and when infected often have lower parasite densities than children [119, 487, 488]. Landmark passive transfer studies have shown that immunoglobulin G purified from adults living in a malaria-endemic area can successfully treat children admitted to hospital with severe malaria[127], suggesting that the mediators of immunity in adults are also functional in children. Identifying the targets and mechanisms by which this anti-disease and anti-parasite immunity is mediated in adults is therefore important and could guide the selection of suitable malaria vaccine candidates.

Immuno-epidemiological studies aimed at identifying the targets of protective immunity against malaria use cohorts typically comprised of children, primarily because they are in the process of acquiring immunity, and can be classified into “protected” and “susceptible” groups, allowing comparison of immune profiles between groups. In contrast, adults in high transmission regions are generally all immune to clinical malaria, and thus of limited value for this kind of approach. However, studying such adults does offer some advantages over studying children. Immunity to clinical episodes in adults is known to be stable in the presence of continuous intense exposure (i.e. there are no outbreaks of clinical malaria reported in adults under these conditions) with exceptions in pregnancy associated malaria [16] and/or immuno-compromised individuals [489, 490]. Similarly, immune responses in adults have been shown to be stable in longitudinally monitored cohorts [491, 492]. By contrast, responses in children can be unstable [491] and have been shown to be short-lived [493], meaning they may be functionally different to adult responses. In addition, retrospective studies on the epidemiological

and clinical manifestations of imported malaria suggest that the clinical immunity gained in adults living in a malaria-endemic area may be long-lived[494]. Thus, unlike children, immune adults are thought to provide a stable clinical and immunological phenotype. I reasoned therefore that a cohort of longitudinally monitored adults living in a highly endemic area of Tanzania was an ideal platform for the identification of potential targets of protective immunity, and used this to monitor responses to the antigens identified in the proteomic studies in Chapters 2-4, as well as previously studied control antigens.

### **5.1. Rationale**

Prospective cohort studies in children are used widely to identify the targets of protective antibodies. This relies on examining antibody responses (sero-positivity or high antibody titers) to a range of antigens in relation to observed clinical episodes of malaria in cohorts of children followed over time. However, the results from such studies have often been contradictory, with protective associations against a given antigen being reported in some studies and contradicted in others from the same or different regions [302]. Many factors could account for this, including differences in i) malaria transmission intensities, ii) methodology and analytical approaches used, iii) the target antigens used given the variations due to antigen polymorphisms. Additional factors specific to the use of serum samples taken from children in these types of analyses contribute further to the discrepancies. Firstly, antibody responses in children have been shown to be short-lived[493] and unstable[491] and therefore a measurement taken before a long follow-up period may not be adequately predictive of protective efficacy. Second, children can easily be misclassified as immune when in reality they were not exposed to infectious mosquitoes during the period of observation. This undermines the ability to clearly distinguish “truly immune” from “susceptible” children and further compromises analyses in cohort studies[495]. Third, even if exposure could be confirmed, children who are immune during one malaria transmission season may be susceptible to malaria during a subsequent season (personal communication, F. Osier).

Examining antibody responses in adults may overcome some of the above limitations, as it is known that almost all adults are immune to clinical episodes of malaria despite living in an area of high transmission intensity. This minimizes the misclassification bias that occurs when defining an immune or susceptible individual. Adult immunity is thought to be stable over time; therefore measurements taken at any time point are likely to be informative with regards to protective immunity. Immune responses measured in longitudinally monitored adults are stable[491, 492], and therefore protective responses should be identifiable at multiple sampling points. I therefore hypothesized that a protective immune response would be detectable in the majority of clinically immune adults (conservative estimate of approximately 50%) at multiple time points.

In addition, studies have shown that the magnitude (titre) of antibody responses [496] as well as the breadth (the number of antigens recognized) [496, 497] is predictive of protection from clinical episodes of malaria in children. I therefore hypothesized further that immune adults living in malaria endemic areas would have high titers of antibodies to a subset of important malaria antigens, and that these titers would be maintained over time.

## **5.2. Objective**

To use sera from a cohort of longitudinally monitored adults to establish an analytical framework for the identification of antigens associated with protective immunity.

## **5.3. Specific objectives**

- Describe the epidemiology of malaria within a cohort of longitudinally monitored immune adults in Nyamisati, Tanzania.
- Identify antibody responses to novel antigens associated with protective immunity in adults

## **5.4. Methods**

### ***5.4.1. Study population: Nyamisati Cohort***

The study was conducted in a longitudinally monitored population in Nyamisati village situated in Rufiji District, coastal Tanzania. Malaria transmission is perennial with seasonal fluctuations and the area is considered to be holoendemic, with a parasite prevalence of > 75 % in 2-9 year olds at the time the cohort was established in 1985 [498-500]. A research team was set up in the village to study the epidemiology of malaria in 1985. Since then, annual cross-sectional surveys have been conducted between March and May before the rainy season and continue to date. The work presented here includes data collected during the 1993, 1994 and 1995 surveys that comprised of 558, 793 and 722 individuals respectively (Figure 5.1). These years were selected as they were conducted during a period of high malaria transmission and both epidemiological data and serum samples were readily available for analysis. During the cross-sectional surveys, venous blood samples were collected and clinical assessments conducted for each individual. All participants were monitored passively for clinical episodes of malaria throughout the year at a single health centre that served the entire village. A malaria episode was defined as a fever (axillary temperature of > 37.5°C), detectable parasites by

microscopy and the absence of clinical findings related to other infections. Individuals who met these criteria were given anti-malarial treatment as per the national guidelines. *Plasmodium* parasites were identified using thick and thin blood films stained with Giemsa. Parasite densities were determined by counting parasites against leucocytes assuming 8000 leucocytes per  $\mu\text{l}$ . One hundred oil immersion fields were examined for microscopy analysis[501]. Data on merozoite surface protein 1 and 2 (MSP1 and MSP2) genotyping [502] as well as a multiplexed real-time polymerase chain reaction (PCR) assay for the detection of the 18S gene [503] was also available for the 1994 cross-sectional survey and for individuals who were 16 years and younger at the 1995 cross-sectional survey. I analyzed the epidemiology of malaria within this cohort and selected individuals who were 15 years and older and asymptomatic at each of the survey (i.e. having no record of malaria at the time of the survey, 4 weeks before or 1 week after) as the adults whose antibody responses would be analyzed. In the individuals in whom a serum sample was available for antibody detection, I also detected the 18S gene in their corresponding packed red cell pellets to provide data on parasite status at the time of the cross-sectional survey.

#### ***5.4.2. Adult immuno-epidemiology: measuring antibody responses to merozoite antigens***

##### ***5.4.2.1. Recombinant merozoite antigens***

Recombinant merozoite antigens used for adult immuno-assays included a panel of well-studied antigens and a subset (11/19, 60%) of the novel antigens described in Chapter 4. The well-studied antigens included; AMA1 (3D7 and FVO alleles), MSP1 (19 kilodalton of the Wellcome strain), MSP2 (CH150/9 and DD2 alleles), MSP3 (3D7-like and K1-like alleles) and RH2 (2030 fragment). These antigens were expressed and purified as has been previously described in the references listed in Table 5.1 with the exception of AMA1. Dr. Ed Remarque of the department of Parasitology Biomedical Primate Research Centre, Netherlands, kindly provided the AMA1 recombinant proteins. The subset of novel antigens identified in this work was selected based on the availability of sufficient quantities of protein to measure



responses in the entire cohort by indirect ELISA. The details of the recombinant antigens expressed and tested in this study are shown in Table 5.1. Full-length AMA1 and MSP2 expressed using the same Expi293 mammalian system used for the novel antigens were included as comparators. Altogether, antibody responses to 21 recombinant proteins were measured in a cohort of longitudinally monitored adults in the Nyamisati cohort, Tanzania.

**Table 5.1: Details of the recombinant *P. falciparum* antigens used in this study**

Recombinant antigen	Region of protein expressed	Purification Tag	Expression system	Immunological assay
MSP-1 <sub>19</sub> [504]	1631-1726	GST	E.coli	Luminex assay
MSP-2 (Dd2)[505]	22-248	GST	<i>E.coli</i>	
MSP-2 (CH150/9)[505]	1-184	GST	<i>E.coli</i>	
MSP-3 (3D7)[170]	2-354	MBP	<i>E.coli</i>	
MSP-3 (K1)[170]	2-379	MBP	<i>E.coli</i>	
AMA-1 (3D7)[506]	83-531	His	<i>P. pastoris</i>	
AMA-1 (FVO)[507]	25-544	His	<i>P. pastoris</i>	
<i>Pf</i> RH2 (2030 fragment)[283]	2030-2528	GST	<i>E.coli</i>	
Antigens expressed in-house using the Expi293 expression system (all are based on the <i>Pf</i> 3D7 strain)				
AMA-1	25-541	His	Expi293 cells	Indirect ELISA
MSP-2	20-246			
PF3D7_0206200( <i>Pf</i> PAT)	241-319			
PF3D7_0629500 (SEG2)	451-544			
PF3D7_0730800.2	20-293			
PF3D7_0823800	93-605			
PF3D7_0830500	55-675			
PF3D7_0925900	21-217			
PF3D7_1025300	18-922			
PF3D7_1252300	2-51			
PF3D7_1308000	341-602			
PF3D7_1345100	24-157			
PF3D7_1401600	69-478			

#### *5.4.2.2. Indirect ELISA to measure antibody responses to novel antigens*

For the eleven novel antigens, a coating concentration for the ELISA was determined as follows: 100µl of recombinant antigen was coated at a two-fold serial dilution of ranging from 128µg/ml to 0.06µg/ml in Dynex 4HBX Immunolon ELISA plates. These were tested with a pool of hyperimmune sera and a negative control as has been described in section 4.4.4. An optimal coating concentration was determined for each antigen. The optimal coating concentration for each antigen was subsequently plated at 100µl per well, diluted in coating buffer (15mM Na<sub>2</sub>CO<sub>3</sub>, 35mM NaHCO<sub>3</sub>, pH 9.4) and left overnight at 4°C. After overnight incubation, plates were washed four times in 1X PBS containing 0.05% Tween-20 (wash buffer) and blocked for 5 hours at room temperature with 1% skimmed milk (Marvel) diluted in PBS Tween 20 (Blocking buffer). Thereafter, plates were washed four times in wash buffer and 100µl of individual sera at a 1:1000 dilution were tested in duplicate. Positive and negative controls were included in each plate as well as a serial dilution of malaria immune globulin (MIG) as a standard positive control and to allow for the conversion of optical density values to relative antibody concentrations in arbitrary units. MIG is a reagent that contains purified immunoglobulin G (98% IgG) from the plasma of 834 healthy Malawian adults[328]. It was originally manufactured to test its potential role as an adjunct therapy for the treatment of cerebral malaria in Malawian children[328]. Following an overnight incubation at 4°C, wells were washed and incubated for 3 hours at room temperature with 100µl of HRP conjugated to polyclonal rabbit anti-human IgG (Dako) at a 1:5000 dilution using block buffer. The wells were then washed four times and incubated at room temperature with 100µl of substrate development buffer (0.1M citric acid, 0.2M Na<sub>2</sub>HPO<sub>4</sub>, 4mg *o*-phenylenediamine dihydrochloride tablets (Sigma), 8µl hydrogen peroxide and 5ml distilled water). After 20 minutes of incubation in the dark, the reaction was stopped with 25µl H<sub>2</sub>SO<sub>4</sub> and absorbance read at 492nm. As a comparator, responses to AMA1 and MSP2 expressed using the same Expi293 expression system were measured as described above. A coating concentration of 0.05µg/ml was used for these antigens [496, 508].

#### *5.4.2.3. Luminex assay to measure antibody responses to the well-studied antigens*

An optimized multiplex bead-based antibody assay that was available in-house [509, 510], was used to measure responses to previously studied merozoite antigens, with the exception of Expi293 expressed MSP2 and AMA1, whose responses were measured by ELISA. Each antigen was coupled to magnetic microsphere beads (Luminex corp) using the Bioplex amine coupling kit (Biorad) according to manufacturer's instructions. I coupled the antigens at a concentration of 1.0 µg/million beads for AMA1 (3D7 and FVO allele) and 0.5 µg/million beads for the other antigens namely MSP1-19, MSP2 (DD2 and CH150/9 allele), MSP3 (3D7 and K1 allele) and RH2-2030. These coating concentrations were determined by performing a checkerboard titration in which both the antigen and serum concentrations were serially diluted to identify optimal assay conditions (Appendix 5.1, Figure 1 and 2). To test for the presence of plasma IgG to the different antigens, the following protocol was used. Antigens coupled to magnetic beads of unique spectral addresses were diluted to a final concentration of 100 beads/µl (b/µl) using 1% BSA/PBS. 50µl of diluted beads (5000 beads) were added to Bio-Plex Pro flat bottom plates (Bio-Rad Laboratories) and 50µl of sera diluted 1:1000 in 1% BSA/PBS was added. The plate was incubated for 1 hour on a microplate shaker (rotation 500 rpm) at room temperature and in the dark. The plate was then washed four times using 0.05% PBS/Tween 20 and 50µl of R-phycoerythrin-conjugated, F(ab')<sub>2</sub> fragment-specific, goat anti-human immunoglobulin G (Jackson ImmunoResearch Laboratories) was added to each well and the plate incubated for 30 minutes at room temperature on the microplate shaker. The plates were washed four times using 0.05% PBS/Tween 20 and each well resuspended in 100µl of 1%BSA/PBS. Each well was then analysed on the MAGPIX using the xPONENT software. The fluorescence intensity was read from at least 100 microspheres per spectral address (corresponding to a single antigen) and reported as mean fluorescence intensity (MFI). Similar to the indirect ELISA controls, a serial dilution of malaria immune globulin were included in each plate as a standard positive control and to allow for the conversion of MFI values to relative antibody concentrations in arbitrary units. A pool of hyper-immune sera (PHIS) drawn from

malaria immune adults in Kenya was also used on each plate to correct for plate-to-plate variations and sera from 30 non-malaria exposed adults were included as negative controls.

### **5.5. Statistical analysis**

All analysis was done using STATA (StataCorp, version 13.1) and Graphpad PRISM (version 6.0h). The Kruskal-Wallis H test for trend was used to analyze the trend between parasite density and age. The Mann-Whitney U test was used to compare median parasite density levels between 0-14 year olds and > 14 year olds treated at the health centre for clinical episodes of malaria. I examined the relationship between antibody responses and age, parasite prevalence and parasite density. Antibody levels were determined from interpolation from the non-linear 5-parameter standard curve made by a tripling dilution of purified malaria-immune IgG that was included in each plate. Antibody levels and parasite densities were log transformed to normalize the data. The cohort of adults was split into two age groups, 15-29 year and > 30 years. The Mann-Whitney U test was used to model associations between antibody levels and age, antibody levels and parasite status (PCR positive versus negative individuals) and antibody levels and clinically symptoms. The Mann-Whitney U test was used to compare median parasite density levels between individuals with a stable versus non-stable antibody response. An individual's breadth score was determined by adding up the number of antigens to which an individual had high antibody titres (above median antibody levels of the cohort). The Kruskal-Wallis test for trend was used to model the breadth score against age and parasite density. The Mann-Whitney U test was used to compare the median breadth score between individual who experienced a clinical episode of malaria versus those who did not.

## 5.6. Results

### 5.6.1. Description of the Nyamisati cohort and selection of clinically immune adults

#### 5.6.1.0. Nyamisati cohort description

I used data collected from three cross-sectional surveys conducted in the years 1993, 1994 and 1995 to describe the epidemiology of malaria in adults living in Nyamisati village, a malaria-endemic region in Tanzania. The epidemiological data that was available at each cross-sectional survey included the year of birth, sex, parasitaemia by microscopy, a description of each individual's house and roofing material, the results of an HIV rapid serology test and confirmatory HIV viral detection assay. PCR data for parasite detection and genotype (as described above) was available for all samples collected at the 1994 survey and for the under 16 year olds in the 1995 survey. PCR data was not available for the 1993 survey. The axillary temperature measurements were conducted for each individual but only recorded in the 1995 survey. Data on the pregnancy status of the females were available for the 1995 survey only.

There were 558, 793 and 722 individuals surveyed at the 1993, 1994 and 1995 surveys respectively (Figure 5.1). These included adults and children whose ages ranged from less than 1 year to 85 years. The data available to calculate the age was the year of birth and therefore an individual's age in months could not be determined. The parasite prevalence in the entire cohort by microscopy was 46.2%, 38.9% and 36.1%, respectively, for each of the three consecutive annual surveys. *Plasmodium falciparum* infection accounted for over 93% of infections in all three surveys with *Plasmodium malariae* accounting for 0.4, 3.3 and 2.4% of infection in the 1993, 1994 and 1995 surveys. There was one case of a mixed *Plasmodium falciparum* and *Plasmodium malariae* infection recorded at the 1993 survey and five (1.7%) cases at the 1994 survey. There were no mixed infections recorded at the 1995 survey. The overall HIV prevalence was low at each of the surveys with an average of 5.1%.

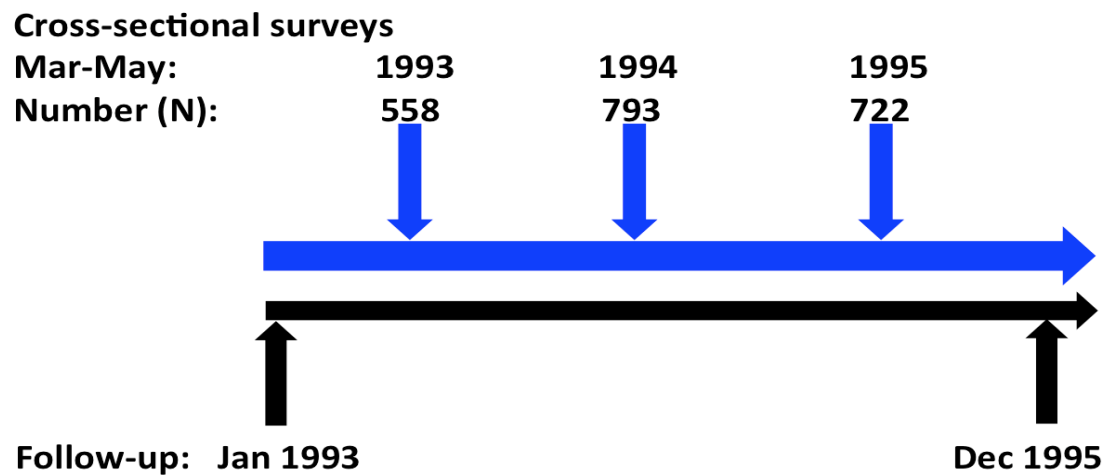


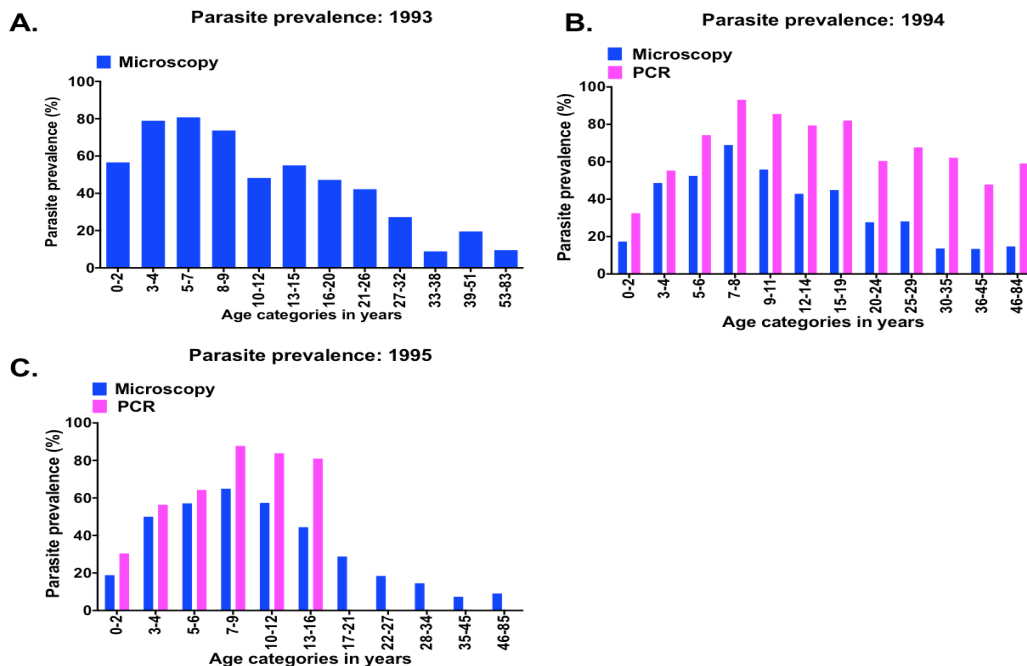
Figure 5.1: Schema showing the three cross-sectional surveys conducted in the Nyamisati village and follow-up period for the cohort of individual's analyzed in this thesis.

#### 5.6.1.1. Parasite prevalence in the Nyamisati cohort

*Plasmodium falciparum* transmission intensity is often estimated using either the *Plasmodium falciparum* parasite rate (*PfPR*) in children aged 2-10 years (*PfPR*<sub>2-10</sub>), which refers to the proportion of the population found to be carrying asexual blood stage parasites in this age group, or the entomological inoculation rate (EIR) that estimates the number of infectious mosquito bites per person per unit time [511]. Data on the EIR was not available for this population and so I used the *PfPR*<sub>2-10</sub> to estimate the transmission intensity in this region at each of the three surveys. The *PfPR*<sub>2-10</sub> was 74%, 62% and 58% as determined by microscopy in the 1993, 1994 and 1995 cross-sectional surveys. The *PfPR*<sub>2-10</sub> estimated using PCR data was 78% and 71% at the 1994 and 1995 surveys respectively. Based on this data, this area would be classified as a region with hyperendemic malaria transmission [511].

A clinical assessment was conducted for all study participants at the time of the cross-sectional survey. Data was available on symptomatic individuals, defined as those who had been on treatment four weeks before, during or one week after the survey. There were 8, 64 and 57 individuals at the 1993, 1994 and 1995 cross-

sectional surveys who were symptomatic. The majority of these individuals were under the age of 10 years: 7 (87.5%), 62 (97.0%) and 52 (91.2%) individuals in the 1993, 1994 and 1995 surveys. To describe the epidemiology of malaria in the cohort, I used data that both included or excluded the symptomatic individuals at each of the cross-sectional surveys. The data presented below excludes the individuals who were symptomatic. As shown in Figure 5.2, the parasite prevalence (microscopy) rose with age reaching a peak of between 65 and 80% at about seven years of age and declining gradually thereafter. The lowest parasite prevalence by microscopy ranged between 7 and 13% and was observed in older adults aged 30 to 85 years. The parasite prevalence estimated by PCR data showed overall higher parasite prevalence in all age groups when compared to microscopy. This was particularly striking for the older age groups (over 6 years olds) as seen in the 1994 (Figure 5.1B) and 1995 (Figure 5.1C) data. For example, using data from the 1994 survey in adults aged between 30-35 years, microscopy underestimated the prevalence of asymptomatic parasitaemia by 48% when compared to the prevalence estimated by PCR data.

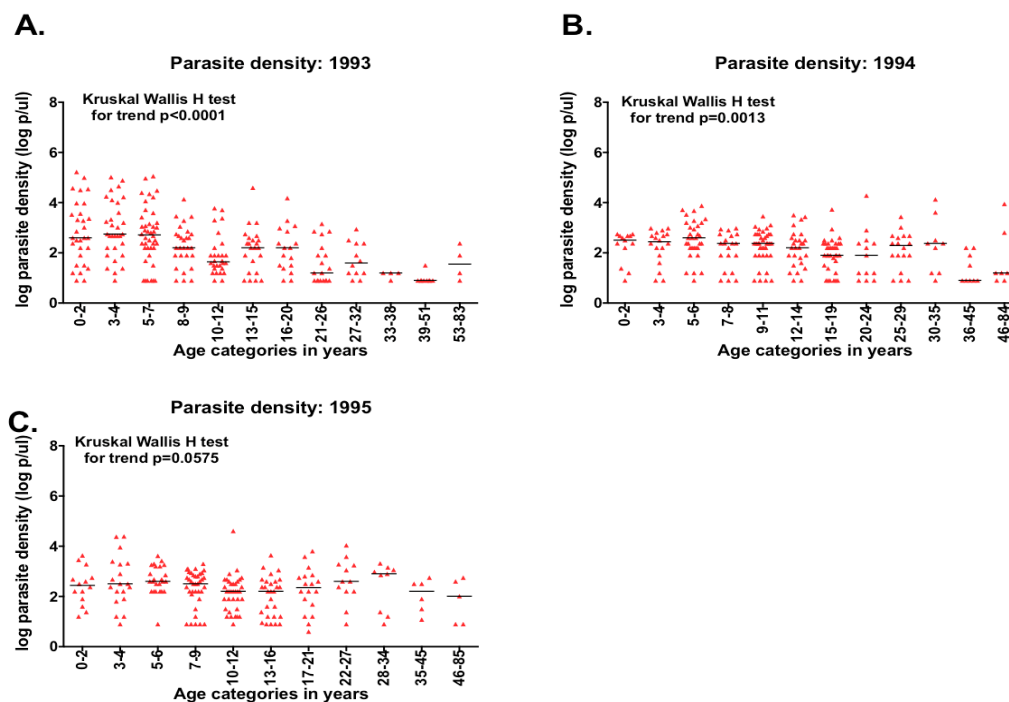


**Figure 5.2: The parasite prevalence by age in the Nyamisati cohort.** The parasite prevalence by age in asymptomatic individuals using data collected at A) 1993 B) 1994 and C) 1995 cross-sectional survey. Blue bars show parasite prevalence determined by microscopy. Purple bars represent parasite prevalence determined by PCR. PCR data is not available for the 1993 survey or for the over 16 year olds in the 1995 survey.

The parasite prevalence in the entire cohort including both symptomatic and asymptomatic individuals is shown in Appendix 5.1, Figure 3.

#### 5.6.1.2. Parasite densities in the Nyamisati cohort

The median parasite density dropped gradually with age in asymptomatic individuals. This decline was statistically significant for the 1993 and 1994 surveys (Figure 5.3A and 5.2B respectively). The highest median parasite densities were recorded in children aged 3-4 years in the 1993 survey (560 parasites/ $\mu$ l, range 8-104,000) and in children aged 5-6 years in the 1994 and 1995 surveys (400 parasites/ $\mu$ l: range 8-7516 and 8-4160 in the 1994 and 1995 surveys respectively). The lowest median parasite densities recorded were 8 (range: 8-32), 8 (range: 8-160) and 100 (range: 8-560) parasites/ $\mu$ l in adults aged 39-51, 36-45 and 46-85 years in the three consecutive surveys.



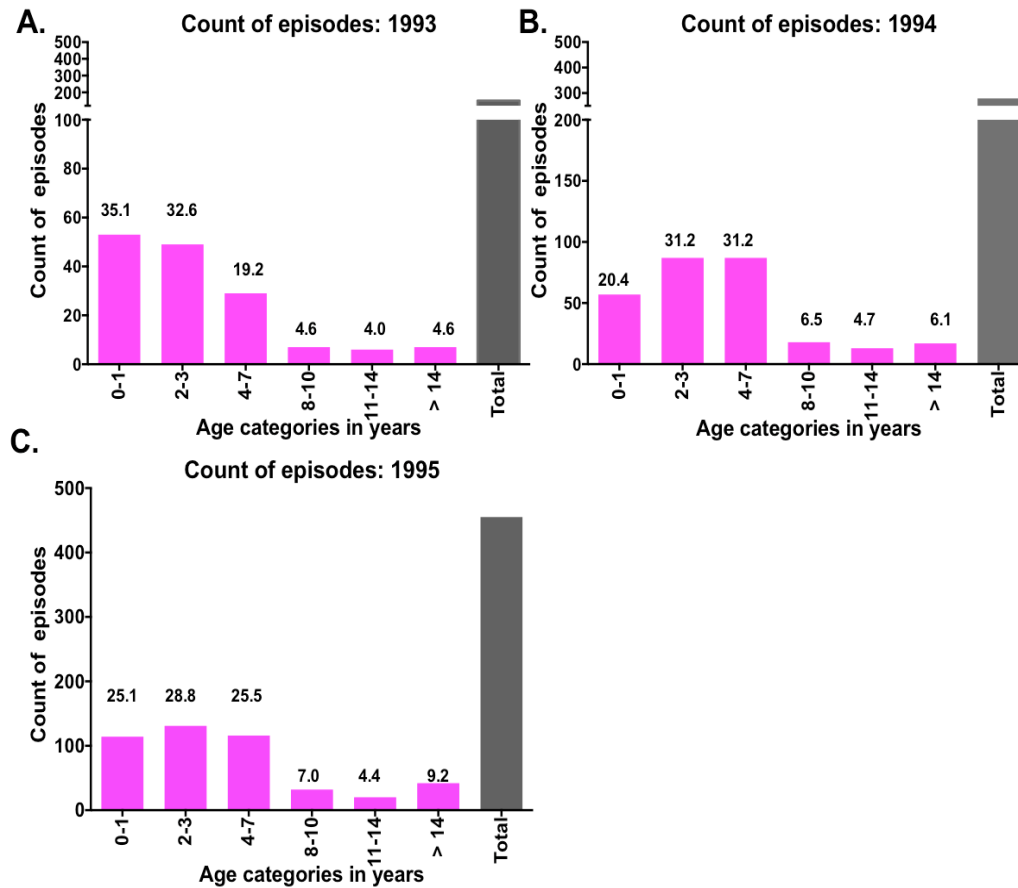
**Figure 5.3: The parasite densities by age in the Nyamisati cohort.** Parasite densities decline with age in asymptomatic individuals. The log<sub>10</sub> parasite density by age using data collected at the A) 1993 B) 1994 and C) 1995 cross-sectional survey. The Kruskal-Wallis H test for trend was used to compare media parasite density levels with age.



The data presented exclude 8, 64 and 57 individuals at the 1993, 1994 and 1995 cross-sectional surveys who were symptomatic at the respective surveys. While the decline in parasite density with age was evident in the 1993 cross-sectional survey, this trend was not observed in the 1995 survey. This may suggest a rapid decline in transmission intensity in the Nyamisati cohort over a 3-year period resulting in concomitant loss of immunity in the population. However, while the *PfPR*<sub>2-10</sub> over the three years dropped from 74-58%, the area would still be classified as experiencing hyperendemic transmission therefore the loss in trend with age is unexpected and loss of immunity within the population is unlikely. An alternative explanation would be that the data collected was not sufficient to make conclusions. The data presented includes parasite densities as measured by microscopy in 46% of the cohort population. Retrospective detection and quantification of parasite densities by PCR would strengthen these findings, however this data was unavailable for analysis at the time of writing this thesis.

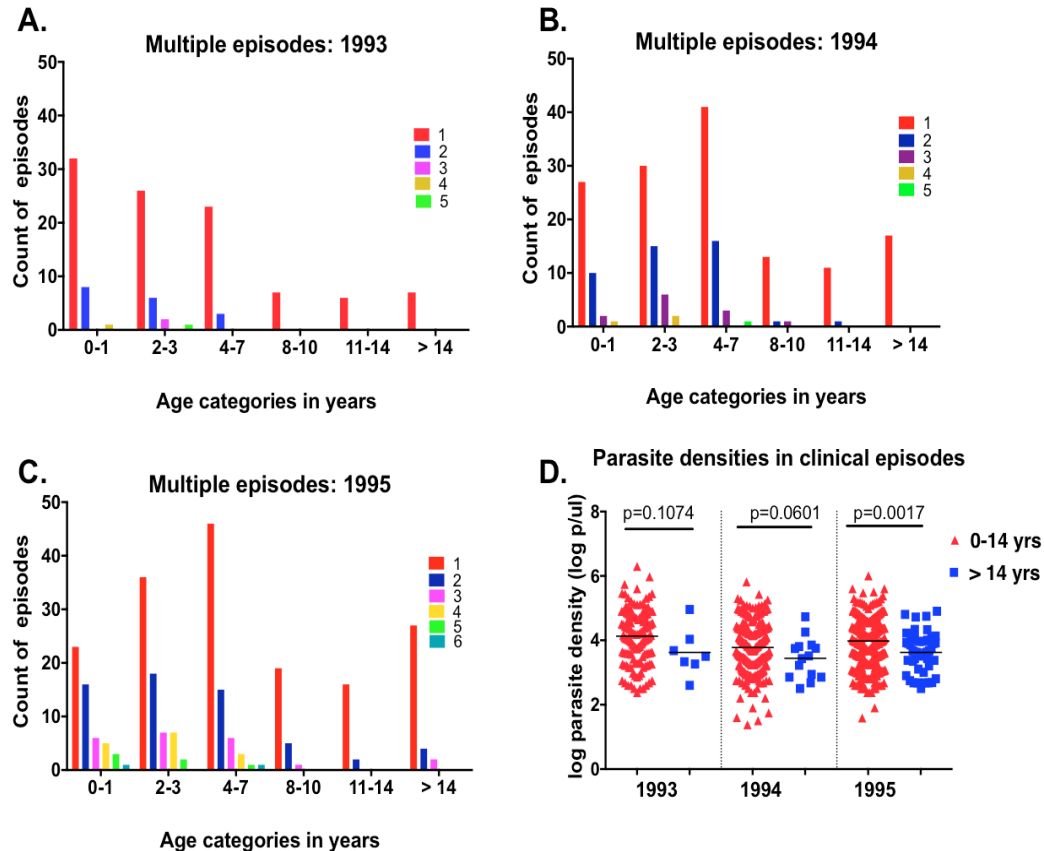
#### *5.6.1.3. Clinical episodes of malaria in the Nyamisati cohort*

Episodes of malaria defined as a fever, detectable parasitaemia by microscopy and the absence of clinical findings related to other infectious diseases were recorded at the health centre. As shown in Figure 5.4, the majority of malaria episodes occurred in children under the age of 8 years, with an average of 83% of all episodes occurring in children aged 0 to 7 years of age. Only 4.6%, 6.1% and 9.2% of recorded malaria episodes occurred in individuals over 14 years in the 1993, 1994 and 1995 respectively. The number of cases varied in each of the cross-sectional surveys resulting in 151, 279 and 455 cases reported at the health centre in the 1993, 1994 and 1995 cross-sectional survey. This probably reflected improvements in diagnosis of malaria as well as data collection and recording in the subsequent years rather than a drop in transmission intensity and immunity that would result in an increase in the clinical episode observed as has been discussed in section 5.6.1.2. For example, while axillary temperature was not available in the 1993 and 1994 data, these data were collected in the 1995 follow-up period. Similarly, data on pregnancy status of the females within the cohort were reported in 1995.



**Figure 5.4: Clinical episodes of malaria decline with age.** The count of episodes by age recorded at the health centre over the A) 1993, B) 1994 and C) 1995 calendar year. Numbers at the top of the column represent the proportion of total episodes.

Multiple episodes per individual were also recorded at the health centre, the majority of which occurred in children under the age of 7 years as shown in Figure 5.5. No individuals over the age of 14 had more than one episode of malaria recorded in the years 1993 (Figure 5.5A) and 1994 (Figure 5.5B). In the year 1995, a total of 7 individuals who were 15 years and older had two or more episodes of malaria recorded at the health centre (Figure 5.5C), and three of these were pregnant women. Five individuals had two recorded episodes of malaria and two individuals had three documented episodes of malaria recorded at the health centre.



**Figure 5.5: Multiple clinical episodes of malaria are rarely experienced in older individuals.** The count of multiple episodes by age recorded at the health centre over the A) 1993, B) 1994 and C) 1995 calendar and D)  $\log_{10}$  parasite densities of individuals treated at the health centre for malaria categorised by age.

The median parasite density of individuals over 14 years and treated for malaria at the health centre ranged between 3000 to 4320 parasites/ $\mu$ l. Interestingly, this parasite density was lower than that observed in children 14 years and younger presenting with clinical malaria at the health centre (6400-14000 parasites/ $\mu$ l) (Figure 5.5D and Table 5.2). This implies that although adults are generally immune, the few who become symptomatic do so at a lower parasite density compared to children. A summary of the demographic characteristics of individuals who were older than 14 years and treated at the health centre for malaria is shown in Table 5.2.

**Table 5.2: Table showing the demographic characteristics of the individuals over 14 years treated at the health centre for a clinical episode of malaria**

Characteristics	1993	1994	1995
No. of episodes recorded	151	279	456
No. of episodes in > 14 year old (%)	7 (4.6)	17 (6.1)	42 (9.2)
Median age in years (min-max)	35 (20-82)	30.5 (16-44)	29 (15-60)
Female	3	8	25
No. pregnant	0	2	5
Median parasite density in > 14 years (min-max)	3200 (400-91200)	3000 (320-54000)	4320 (320-6400)
Mean axillary temperature (min-max)	nd	nd	37.5 (36.0-40.20)
<i>Plasmodium falciparum</i> infection	6	17	42
Mixed infections	1 ( <i>Pf</i> + <i>Pv</i> )	0	0
Median parasite density in < 15 year old (min-max)	14000 (240-19800)	6400 (40-660000)	11600 (80-400000)

Based on these data, I selected individuals who were over 14 years and asymptomatic at the time of the 1993 survey (i.e. those who had not been on treatment four weeks before, during or one week after) for immuno-epidemiology assays. As it was a longitudinal study spanning three years, I limited analysis to adults with a sample available in all three cross-sectional surveys, and who were asymptomatic at the time of sampling at all three time points. A summary of the demographic details of these selected adults at each cross-sectional survey is shown in Table 5.3.

**Table 5.3: Table showing the demographic characteristics of the individuals over 14 years whose antibody responses were measured.**

<b>Characteristics</b>	<b>1993</b>	<b>1994</b>	<b>1995</b>
Total number of individuals	558	793	722
Number of individuals aged over 14 years	283	388	353
Number of samples available (%)	77		
Median age in years (Min-Max)	33 (15-78)	34 (16-79)	35(17-80)
Females (%)	46(59.7)		
Pregnant (%)	nd*	nd*	5(10.8)
HIV positive (%)	0		
Parasite prevalence (Microscopy)	20 <sup>#</sup> (26.3)	12 <sup>#</sup> (15.8)	8 <sup>#</sup> (10.5)
Parasite prevalence (PCR)	28 <sup>&amp;</sup> (41.2)	40 <sup>%</sup> (54.8)	31 <sup>@</sup> (43.7)
Median parasite density (p/μl) (Min-Max) in parasite positive individuals by microscopy	16 (8-1200)	16 (8-240)	24(8-640)

\*: No data available. #: N=76. &: N=68. %: N=73. @: N=71

### ***5.6.2. Antibody responses in a longitudinally monitored cohort of adults***

I measured antibody responses to 21 recombinant antigens, which were obtained using different expression systems as shown in Table 5.1. Antibody responses to a subset of the antigen were measured using the Luminex assay and the remainder by indirect-ELISA for logistical reasons.

#### ***5.6.2.1. Comparison between antibody responses to antigens recombinantly expressed using multiple systems***

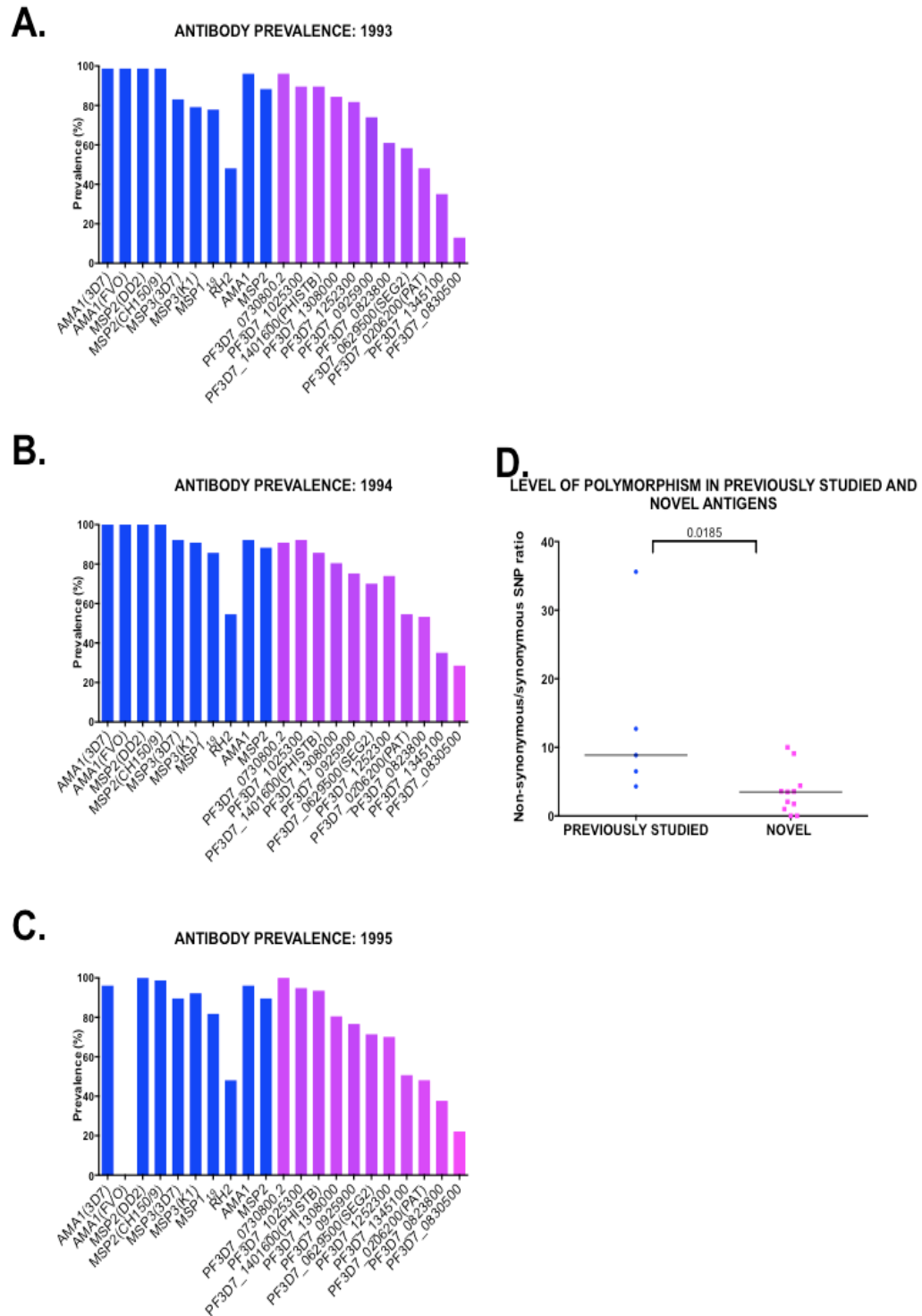
The majority of the previously studied antigens analysed in this study were obtained using the *E.coli* or *P. pastoris* heterologous expression while the novel antigens were expressed using an Expi293 mammalian expression system as has been described in Chapter 4. In addition, antibody responses to well-studied antigens were measured using the Luminex assay at the beginning of the study while responses to novel antigens were measured using an indirect ELISA (Table 5.1). Therefore it was important to see that antigens expressed in the different systems gave comparable results and were positively correlated.

To compare the ELISA and Luminex immuno-assays, I tested 72 serum samples for the presence of IgG antibodies to the same antigens namely: AMA1-3D7 and MSP2-CH150/9, using a well established in-house ELISA assay [496] and an optimized multiplex Luminex assay based on the parameters described in section 5.4.2.3 and Appendix 5.1, Figure 1 and 2. A standard curve made from a tripling dilution of purified IgG from adults in Malawi [328] was included in each plate and used to interpolate antibody concentrations for each tested sample. As shown in Appendix 5.1, Figure 5, a strong positive Pearson correlation coefficient was observed for each antigen indicating that the optimised multiplex Luminex assay and the monoplex ELISA assay were comparable. Next, I compared antibody responses in the same serum samples to AMA1-3D7 and MSP2-CH150/9 expressed in different expression systems (Table 5.1). The full-length ecto-domains of AMA1 and MSP2 were expressed using the Expi293 mammalian expression system described in Chapter 4. These were compared to responses measured against AMA1

expressed using *P. pastoris* and MSP2 expressed in *E. coli* that were available in-house. As shown in Appendix 5.1, Figure 6, a strong positive Pearson correlation coefficient was observed for antibody responses to AMA1. Responses to MSP2 were also positively correlated, albeit to a lesser extent than for AMA1, perhaps indicating differences in folding efficiencies between the two systems (Appendix 5.1. Figure 6 C and D).

#### *5.6.2.2. Prevalence of antibodies to known and novel antigens defined by seropositivity*

To determine the prevalence of antibody responses to merozoite antigens, a seropositivity cut-off was determined using 30 non-exposed sera from Swedish adults. A sample with an MFI or OD value greater than the cut-off defined as the mean + 3 standard deviation values of the 30 serum samples from non-exposed adults was interpreted as being positive. As shown in Figure 5.6, the prevalence of antibodies to the novel antigens ranged from as low as 10% to 100% across each of the three cross-sectional surveys. The prevalence of antibodies to PF3D7\_0730800.2, PF3D7\_1025300, PHISTb and PF3D7\_1308000 were over 80% across all three cross-sectional surveys, similar to the prevalence of antibodies to previously studied merozoite antigens AMA1, MSP2 and -3. Antibody prevalence to PF3D7\_0925900 and PF3D7\_1252300 were over 70% while those to PF3D7\_1345100, PfPAT and PF3D7\_0830500 was less than 55%. The prevalence of antibodies to PF3D7\_0925900 ranged between 58-72% while those to PF3D7\_0823800 ranged between 38-61%. The antibody prevalence to PF3D7\_0830500 was the lowest and ranged from 13 in 1993 to 28% in the 1994 cross-sectional surveys.



**Figure 5.6: Antibody prevalence to novel antigens.** Antibody prevalence and polymorphism levels to five known merozoite antigens (blue bars) and eleven novel antigens (purple bars) at the A) 1993, B) 1994 and C) 1995 cross-sectional survey. D) A comparison of the non-synonymous/synonymous SNP ratio (dN/dS) between previously studied antigens (blue circles) and novel antigens (purple squares).



#### 5.6.2.4. Antibody responses to known and novel antigens by age and parasite status

I measured antibody responses to adults whose ages ranged from 15 years to 80 years (Table 5.3). I categorized the individuals into two age categories, those between 15 to 29 years and those 30 years and older. As shown in Table 5.4, antibody responses did not vary with age for all tested antigens. This is in contrast to responses in children whose levels are known to increase with increasing age. However, antibody levels were generally higher in PCR positive individuals compared to PCR negative individuals for all tested antigens (Table 5.5) suggesting that antibody levels were boosted in the presence of parasites.

**Table 5.4: Association between antibody levels and age**

Antigen	1993 <sup>#</sup>		1994 <sup>%</sup>		1995 <sup>*</sup>	
	Z-score	P-value	Z-score	P-value	Z-score	P-value
MSP-1 <sub>19</sub>	0.218	0.8273	-0.355	0.7222	-0.360	0.7188
MSP-2 (Dd2)	0.748	0.4545	0.323	0.7466	0.883	0.3773
MSP-2 (CH150/9)	1.330	0.1837	0.592	0.5536	0.105	0.9167
MSP-3 (3D7)	-0.664	0.5196	-0.657	0.5111	-0.523	0.6011
MSP-3 (K1)	1.039	0.2990	-0.786	0.4317	-0.105	0.9167
AMA-1 (3D7)	1.662	0.0965	1.266	0.2056	0.534	0.5931
AMA-1 (FVO)	1.402	0.1608	1.260	0.2076	nd	
<i>Pf</i> RH2 (2030 fragment)	0.862	0.3886	0.571	0.5681	0.372	0.7101
Antigens expressed in-house using the Expi293 expression system (all are based on the <i>Pf</i> 3D7 strain)						
AMA-1	1.413	0.1578	0.937	0.3487	0.395	0.6929
MSP-2	1.002	0.3162	0.679	0.4974	0.523	0.6011
PF3D7_0206200( <i>Pf</i> PAT)	-0.987	0.3281	-1.497	0.1343	-0.592	0.5535
PF3D7_0629500 (SEG2)	-0.156	0.8762	-0.765	0.4444	0.761	0.4467
PF3D7_0730800.2	-0.327	0.7435	-0.856	0.3918	-0.511	0.6092
PF3D7_0823800	-0.374	0.7084	-0.097	0.9228	0.523	0.6011
PF3D7_0830500	0.977	0.3288	-0.657	0.5111	1.197	0.2315
PF3D7_0925900	-0.426	0.6702	-0.631	0.5285	0.651	0.5153
PF3D7_1025300	-0.561	0.5749	-0.673	0.5008	-1.336	0.1816
PF3D7_1252300	1.698	0.0894	0.485	0.6287	1.162	0.2454
PF3D7_1308000	-0.805	0.4208	-1.734	0.0829	-0.883	0.3773
PF3D7_1345100	0.473	0.6365	-0.668	0.5042	0.732	0.4643
PF3D7_1401600	0.769	0.4421	-0.991	0.3217	0.232	0.8163

#: 1993: Individuals 30 years and older =46 (60%). %: 1994: Individuals 30 years and older =51 (66%). \*: 1995: Individuals 30 years and older =57 (74%).

**Table 5.5: Association between antibody levels and parasite status at the cross-sectional survey**

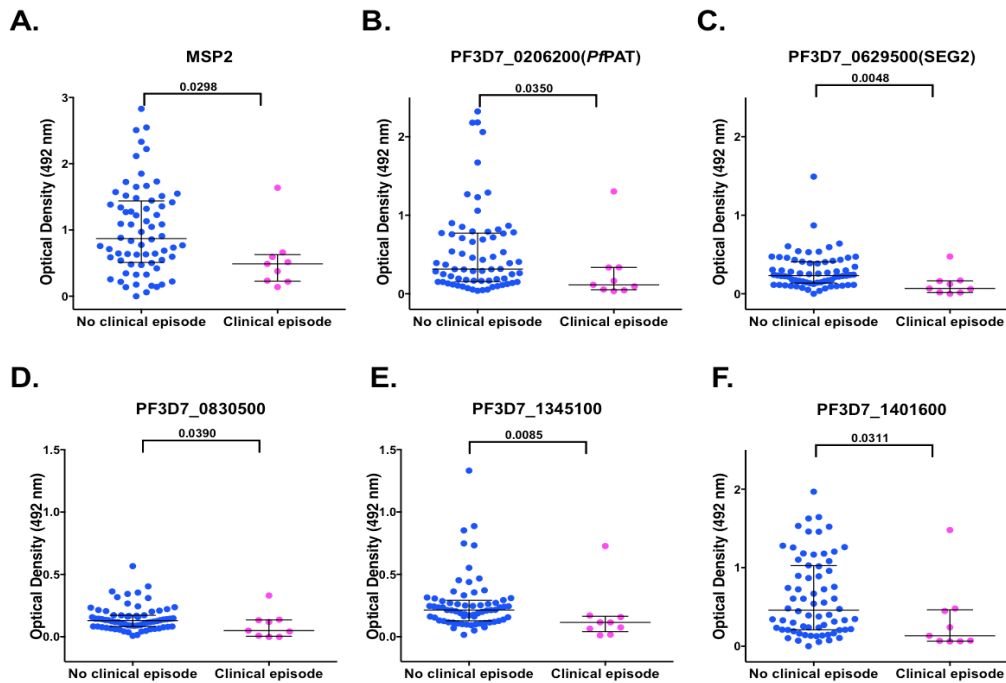
Antigen	1993 <sup>#</sup>		1994 <sup>%</sup>		1995 <sup>*</sup>	
	Z-score	P-value	Z-score	P-value	Z-score	P-value
MSP-1 <sub>19</sub>	-1.620	0.1053	-0.299	0.7647	-0.267	0.7897
MSP-2 (Dd2)	<b>-2.617</b>	<b>0.0089</b>	<b>-2.339</b>	<b>0.0194</b>	-1.507	0.1318
MSP-2 (CH150/9)	-0.860	0.3899	-1.452	0.1465	-1.113	0.2657
MSP-3 (3D7)	-1.545	0.1223	-0.344	0.7311	-0.904	0.3658
MSP-3 (K1)	-1.470	0.1415	-0.732	0.4644	<b>-2.133</b>	<b>0.0329</b>
AMA-1 (3D7)	-1.034	0.3010	-1.208	0.2270	-0.487	0.6263
AMA-1 (FVO)	-1.470	0.1415	-1.164	0.2445	nd	
<i>Pf</i> RH2 (2030 fragment)	-1.445	0.1483	-0.599	0.5495	-0.580	0.5620
Antigens expressed in-house using the Expi293 expression system (all are based on the <i>Pf</i> 3D7 strain)						
AMA-1	-0.386	0.6993	-1.097	0.2726	-0.243	0.8076
MSP-2	-1.271	0.2037	<b>-2.361</b>	<b>0.0182</b>	-0.580	0.5621
PF3D7_0206200( <i>Pf</i> PAT)	-0.847	0.3968	-0.111	0.9117	-0.046	0.9630
PF3D7_0629500 (SEG2)	<b>-3.022</b>	<b>0.0025</b>	-1.319	0.1872	-0.985	0.3244
PF3D7_073800.2	-1.707	0.0878	<b>-1.906</b>	<b>0.0566</b>	0.012	0.9907
PF3D7_0823800	<b>-2.729</b>	<b>0.0064</b>	-1.097	0.2725	-0.151	0.8802
PF3D7_0830500	<b>-2.237</b>	<b>0.0253</b>	-0.449	0.6535	-0.603	0.5466
PF3D7_0925900	-0.966	0.3341	0.011	0.9911	-0.475	0.6345
PF3D7_1025300	-1.863	0.0625	-0.233	0.8159	-1.264	0.2063
PF3D7_1252300	-1.751	0.0800	<b>-2.411</b>	<b>0.0159</b>	-0.394	0.6934
PF3D7_1308000	<b>-2.374</b>	<b>0.0176</b>	-0.964	0.3349	0.185	0.8528
PF3D7_1345100	<b>-2.773</b>	<b>0.0056</b>	-0.410	0.6817	0.603	0.5466
PF3D7_1401600	-1.371	0.1704	<b>-2.178</b>	<b>0.0294</b>	-0.707	0.4794

#: 1993: Individuals who were parasite positive (PCR detection of 18S gene)=28(41.2%). %: 1994: Individuals who were parasite positive (PCR detection of 18S gene)=40(54.8%). \*: 1995: Individuals who were parasite positive (PCR detection of 18S gene) =31(43.7%). Significant differences are shown in bold.

#### 5.6.2.5. Association between antibody responses to novel antigens and clinical episodes of malaria

As shown in Figure 5.4 and Table 5.2, the majority of clinical episodes of malaria recorded over the follow-up period were reported in children under the age of 7 years. Less than 10% of the episodes recorded occurred in individuals 15 years and older. In the 77 individuals whose antibody responses were measured, 3 of them experienced a clinical episode in 1994 and 9 individuals experienced an episode in 1995. No episodes of malaria were recorded in the year 1993 in these 77 individuals. The age and haemoglobin levels between individuals who had a febrile

malaria episode did not differ from those who did not, as shown in Appendix 5.1, Figure 7. I then compared the total IgG antibody titres to each antigen in individuals who did or did not experience a clinical episode of malaria in the 1995 follow-up period (68 versus 9 individuals, respectively). In general, antibody titres were higher in clinically immune individuals and the difference was statistically significant for five of the novel antigens tested. RH2 was the only exception in which titres were higher in the individuals who experienced a clinical episode of malaria (Table 5.6, Appendix 5.1, Figure 8). As shown in Figure 5.7 and Table 5.6, antibody levels to 5 (45%) novel antigens were significantly higher in adults who did not experience a clinical episode of malaria compared to those who did. These antibodies were collected in Mar-May, 1995 prior to the occurrence of the clinical episodes evaluated. These antigens were *PfPAT*, PF3D7\_0629500, PF3D7\_0830500, PF3D7\_1345100 and PF3D7\_1401600(PHISTb). This was similar to results observed with MSP2 (Figure 5.7A).



**Figure 5.7: Antibody levels to novel antigens were lower in adults who experienced a clinical episode of malaria.** Lower antibody levels to five novel antigens in adults who experienced a clinical episode of malaria. A comparison of the antibody levels in individuals who experienced a clinical episode of malaria versus those who did not to A) MSP2 B) PF3D7\_0206200 C) PF3D7\_0629500 D) PF3D7\_0830500 E) PF3D7\_1345100 and F)

PF3D7\_1401600. Blue circles represent antibody levels in individuals who did not experience a clinical episode while purple circles represents individuals who did.

I then analyzed the association between antibody responses to individual antigens defined as seropositivity and clinical episodes of malaria. Antibody seropositivity was fitted as the independent variable in a logistic regression model separately for each individual antigen. The dependent variable was whether or not a clinical episode was experienced over the 1-year follow-up period. Antibody responses to all the novel antigens measured were associated with reduced odds of experiencing a clinical episode (Table 5.6).

**Table 5.6: Association between antibody levels and protection from clinical episodes of malaria over the 1995 follow-up period.**

Antigen	Median antibody levels			Associations with the risk of malaria		
	No clinical episode N=68	Clinical episode N=11	P-value <sup>\$</sup>	Prevalence N (%)	Odds ratio (95% CI) <sup>#</sup>	P-value <sup>#</sup>
MSP-1 <sub>19</sub>	2909	1086	0.0551	63(81.8)	0.33 (0.07-1.60)	0.170
MSP-2 (Dd2)	3129	1443	0.1582	77 (100)	nd	
MSP-2 (CH150/9)	824.9	595.4	0.9494	76 (98.7)	nd	
MSP-3 (3D7)	597.7	303.8	0.5259	69 (89.6)	0.38 (0.06-2.28)	0.289
MSP-3 (K1)	514.5	233.1	0.3496	71 (92.2)	0.58(0.06-5.85)	0.647
AMA-1 (3D7)	4526	2426	0.1320	74 (96.1)	nd	
<i>Pf</i> RH2 (2030 fragment)	416.2	613.3	0.6686	37(48.1)	2.60 (0.59-11.43)	0.207
AMA-1	2.424	2.147	0.2104	74 (96.1)	0.21 (0.02-2.85)	0.243
<b>MSP-2</b>	<b>0.871</b>	<b>0.488</b>	<b>0.0298</b>	<b>69 (89.6)</b>	0.93 (0.10-8.68)	0.946
<b>PF3D7_0206200(<i>Pf</i>PAT)</b>	<b>0.314</b>	<b>0.114</b>	<b>0.0350</b>	<b>37 (48.1)</b>	0.48 (0.11-2.11)	0.331
<b>PF3D7_0629500 (SEG2)</b>	<b>0.231</b>	<b>0.069</b>	<b>0.0048</b>	<b>55 (71.4)</b>	<b>0.16 (0.03-0.70)</b>	<b>0.015</b>
PF3D7_0730800.2	0.705	0.427	0.1320	77 (100)	nd	
PF3D7_0823800	0.133	0.163	0.5953	29 (37.7)	0.48 (0.08-2.29)	0.329
<b>PF3D7_0830500</b>	<b>0.130</b>	<b>0.049</b>	<b>0.0390</b>	<b>17 (22.1)</b>	0.39 (0.04-3.39)	0.395
PF3D7_0925900	0.116	0.057	0.1881	59 (76.6)	0.33 (0.08-1.42)	0.137
<b>PF3D7_1025300</b>	1.030	0.267	0.1491	<b>74 (94.8)</b>	<b>0.10 (0.01-0.88)</b>	<b>0.038</b>
PF3D7_1252300	0.220	0.104	0.1165	54 (70.1)	0.29 (0.07-1.25)	0.097
PF3D7_1308000	0.372	0.407	0.3578	62 (80.5)	0.45 (0.09-2.08)	0.309
<b>PF3D7_1345100</b>	<b>0.214</b>	<b>0.115</b>	<b>0.0085</b>	<b>39 (50.7)</b>	<b>0.10 (0.01-0.89)</b>	<b>0.038</b>
<b>PF3D7_1401600(PHISTb)</b>	<b>0.460</b>	<b>0.132</b>	<b>0.0311</b>	<b>72 (93.5)</b>	<b>0.06 (0.01-1.45)</b>	<b>0.006</b>

<sup>\$</sup>=Mann-Whitney U test was used to compare antibody titres in individuals who experienced a clinical episode versus those who did not. <sup>#</sup>=Associations between responses to individual antigens and the odds of malaria were analyzed using a logistic regression model. Seropositivity was used for analysis. Odds ratios are adjusted for age. nd= odds ratio not computed as seropositivity for this antigen was > 96%. Statistically significant associations are shown in bold.

These associations were statistically significant for four antigens namely PF3D7\_0629500, PF3D7\_0830500, PF3D7\_1345100 and PF3D7\_1401600(PHISTb). Interestingly, although the numbers are small, significant associations were not observed for the previously studied antigens (Table 5.6). To adjust for multiple comparisons in the above analysis, the Bonferroni correction was applied resulting in a readjusted statistical significance level of P-value=0.0025. Based on this criterion, the presence of antibodies to PF3D7\_1401600 (PHISTb) remained significantly associated with reduced odds of clinical episodes of malaria.

#### *5.6.2.6. Stability of antibody responses to novel antigens*

The antibody prevalence to novel targets ranged from as low as 13% (PF3D7\_0830500) to as high as 100% (PF3D7\_0730800.2). I therefore explored the stability of a seropositive or seronegative response to the novel antigens in immune individuals in order to test the hypothesis that antigens likely to be important for immunity were stable in a majority of adults. I excluded 11 individuals who experienced a clinical episode of malaria in the entire 3-year follow-up period. As shown in Figure 5.8, responses in individuals were generally stable i.e. individuals who were seropositive at one time point tended to remain seropositive at subsequent time points for most antigens. This was particularly evident in PF3D7\_0206200, PF3D7\_0629500, PF3D7\_0823800, PF3D7\_0830500 and PF3D7\_1345100, where the same individuals tended to be positive at measurements made in subsequent surveys. Antibody seropositivity to previously studied merozoite antigens AMA1, MSP-2, -3 and RH2 were also stable in the three crosssectional surveys (Appendix 5.1, Figure 9).

I then compared the parasite densities measured at each of the three cross-sectional surveys in individuals with stable responses versus non-responders. These two categories were defined as follows for novel antigens; a) stable responders were defined as individuals with a seropositive response in at least two of the three crosssectional surveys and b) non-responders were individuals who were

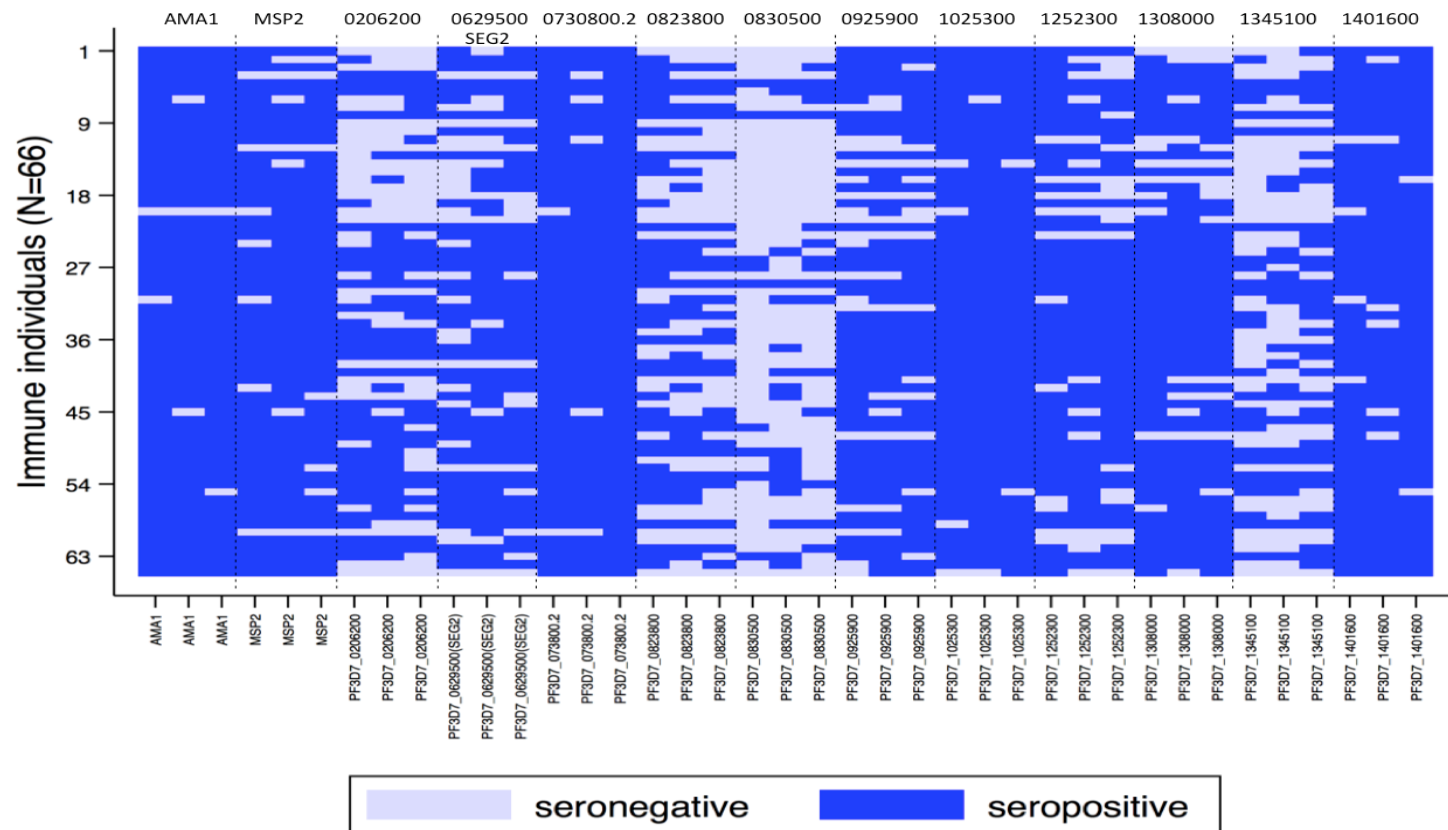
seronegative at all three cross-sectional surveys or had a seropositive response in only one of the surveys. For two of the novel antigens namely PF3D7\_0730800.2 and PF3D7\_1401600, the stable seropositivity rate was over 95% limiting comparisons between responders and non-responders (Table 5.7). For these antigens, the median antibody titres were used as a second cut-off. Stable responders to these two antigens were defined as individuals with a response above the median titre in at least two of the three cross-sectional surveys and non-responders were individuals in whom the titres were below median levels at all three cross-sectional surveys or had an high-titre response in only one of the surveys. As a comparison, I compared parasite densities to previously studied antigens (AMA1, MSP-1<sub>19</sub>, -2, -3 and RH2) using the same criteria as above based on both seropositivity and median antibody levels as the cut-off. As shown in Appendix 5.1, Figure 10, responses to AMA1 (3D7 and FVO alleles) and MSP3 (3D7 and K1 alleles) were highly correlated and suggesting cross-reactivity, while MSP2 induced a more obvious allele-specific response. I therefore used only the 3D7 allele for AMA1 and MSP3 and included both alleles of MSP2 in the analyses below.

The prevalence of stable responders (seropositivity or above median levels) varied greatly between antigens ranging from a low of 18% to PF3D7\_0830500 to a high of 92% to PF3D7\_1025300. Over 50% of the adults tested had stable responses to six of the novel antigens tested in this study. There was a modest but statistically significant lower median parasite densities in individuals with a stable seropositive response to PF3D7\_1308000 compared to those who were not stable (Table 5.7). Median parasite densities were also lower in individuals with a stable seropositive response to most novel antigens with the exception of PF3D7\_1252300, although none of these differences were statistically significant. Interestingly, parasite densities (as determined by microscopy) were higher in individuals with a stable high titre (above median responses) to AMA1 (Table 5.5).

**Table 5.7: Association between stable antibody responses and parasite densities.**

Antigen	Proportion of stable responders N (%)	Median parasite densities (parasites/ $\mu$ l)		Z-score <sup>\$</sup>	P-value <sup>\$</sup>
		Non-responders	Stable responders		
MSP-1 <sub>19</sub>	<sup>b</sup> 38 (49.4)	32	32	0.584	0.5589
MSP-2 (Dd2)	<sup>b</sup> 35 (45.5)	32	32	-0.963	0.3354
MSP-2 (CH150/9)	<sup>b</sup> 36 (46.8)	32	24	0.863	0.3882
MSP-3 (3D7)	<sup>b</sup> 39 (50.7)	16	32	0.549	0.5832
AMA-1 (3D7)	<sup>b</sup> <b>40 (52)</b>	<b>16</b>	<b>88</b>	<b>-2.622</b>	<b>0.0087</b>
<i>Pf</i> RH2 (2030 fragment)	<sup>b</sup> 38 (49.4)	16	32	-0.524	0.6003
AMA-1	<sup>a</sup> 75 (97.4)	nd			
	<sup>b</sup> <b>41 (53.3)</b>	<b>14</b>	<b>96</b>	<b>-2.660</b>	<b>0.0078</b>
MSP-2	<sup>a</sup> 72 (93.5)	nd			
	<sup>b</sup> 36 (46.8)	32	32	0.261	0.7942
PF3D7_0206200( <i>Pf</i> PAT)	38 (49.4)	28	32	0.484	0.6286
PF3D7_0629500 (SEG2)	55 (71.4)	88	32	0.986	0.3244
PF3D7_073800.2	<sup>a</sup> 75 (97.4)	nd			
	<sup>b</sup> 36 (46.8)	80	16	1.707	0.0878
PF3D7_0823800	37 (48.1)	51	32	0.8606	0.4202
PF3D7_0830500	14 (18.2)	32	32	0.484	0.6286
PF3D7_0925900	60 (77.9)	24	32	0.164	0.8700
PF3D7_1025300	71 (92.2)	49	32	0.163	0.8703
PF3D7_1252300	61 (79.2)	25	32	-0.449	0.6534
<b>PF3D7_1308000</b>	<b>65 (84.4)</b>	<b>604</b>	<b>32</b>	<b>2.286</b>	<b>0.0223</b>
PF3D7_1345100	27 (35.1)	32	32	0.528	0.5972
PF3D7_1401600(PHISTb)	<sup>a</sup> 73 (94.8)	nd			
	<sup>b</sup> 39 (50.7)	51	32	0.644	0.5198

<sup>\$</sup>=Mann-Whitney U test was used to compare log<sub>10</sub> parasite densities recorded in all three cross-sectional survey in individuals who were defined as stable responders or non-responders. a=Stable responders were defined as individuals with a seropositive response in atleast two of the three crossectional surveys. b= stable responders in these cases, were defined as individuals with a response above the median titre in atleast two of the three crossectional surveys. Statistically significant differences are shown in bold.



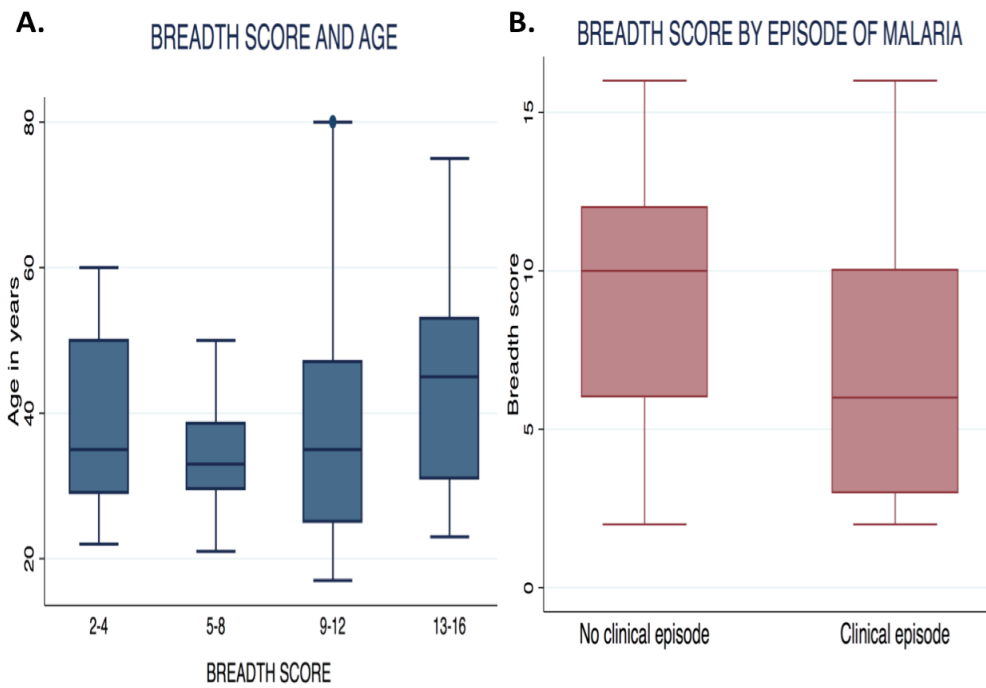
**Figure 5.8: Stability of seropositive response in adults.** The majority of immune adults have stable seropositive response to novel antigens. Plot matrix showing the seropositive response to AMA1, MSP2 and eleven novel antigens, in 66 clinically immune individuals with samples collected at the 1993, 1994 and 1995 cross-sectional survey. Each row on the Y-axis represents an individual while the X-axis shows responses to each antigen grouped together and arranged in consecutive years. For example, for AMA1, the three consecutive columns represent 1993,1994 and 1995 seropositive response.



#### 5.6.2.7. *Breadth of antibody responses to novel antigens*

The breadth of antibody response, defined as the sum of high titer responses to individual merozoite antigens has been previously shown to be a strong predictor of immunity against clinical episodes of malaria in children [496]. To determine the breadth of antibody response in each individual, a single allele was included in the analysis for all antigens, resulting in a total of 16 measurements considered (five previously studied targets and eleven novel antigens). A stable response for the previously studied targets and two novel antigens (PF3D7\_1401600 & PF3D7\_0730800.2) was defined as a response above the median titre in at least two of the three cross-sectional surveys. For the remaining targets (9/11 novel targets) a stable response was defined as having a seropositive response in at least two of the three cross-sectional surveys. I then determined the breadth score by adding up the number of antigens to which an individual had a stable seropositive response. For example, an individual with a breadth score of 16 would have had a stable seropositive response for 9 of the antigens tested and a high titre response (above median antibody levels) to PF3D7\_073800.2, PF3D7\_1401600 and the 5 previously studied antigens ( $9+2+5=16$ ).

Using this definition, no adult had a breadth score of 0. The lowest score obtained was 2 in 3 (4%) of the individuals tested. Similarly 3 individuals had a stable response to all antigens measured resulting in a breadth score of 16 (Appendix 5.2). I categorized the individuals into 4 groups based on their breadth score (2-4, 5-8, 9-12 & 13-16). As shown in Figure 5.9A, a trend of an increase in breadth score with increase in age was observed although this was not statistically significant. Interestingly, the median breadth score was higher in clinically immune adults compared to those who experienced a clinical episode over the three year follow-up period (no clinical episode versus clinical episode breadth score: 10 versus 6:  $p$ -value=0.047)(Figure 5.9B).



**Figure 5.9: The breadth of stable antibody responses in adults by A) age and B) clinical status.**

## 5.7. Discussion

### *Novel targets of naturally acquired humoral responses*

The main aim of this study was to identify novel antigens associated with protective immunity that could feed into the malaria vaccine development pipeline. Although the numbers of serum samples tested are small, the work in this chapter provides evidence that suggests these novel antigens are targets of naturally acquired humoral responses and six of them appear to be associated with protection from clinical episodes of malaria in adults. The antibody prevalence to the panel of eleven novel antigens varied from a low of 13% to 100%, the majority of which were lower than the prevalence observed for previously studied merozoite antigens that are under evaluation as leading vaccine candidates. Encouragingly, the prevalence to the previously studied merozoite antigens tested in this panel was similar to those observed in adults living in high malaria transmission settings such as Senegal[512], western[513] and coastal Kenya[163, 168]. While antibody levels did not increase with age, they were boosted in the presence of parasites as has been observed in children[496, 510, 514]. The responses to previously studied and novel antigens in adults were stable when measured in the same individuals in subsequent surveys similar to observations in Gambian and Kenyan adults[491, 492].

### *Novel antigens associated with protection from clinical episodes of malaria*

Antibody responses to six (66%) of the novel antigens tested were individually associated with protection from clinical episodes of malaria in adults. For the majority of these targets (4/6), the responses were defined as seropositive/seronegative suggesting that a low antibody concentration may be sufficient for protection. Over 50% of adults had stable responses to four of the targets namely PfPAT, PF3D7\_0629500, PF3D7\_1025300 and PF3D7\_1401600(PHISTb) across three surveys. The breadth of antibody responses has also been shown to be associated with protection from clinical malaria in children [496, 510]. I observed that in adults, the breadth score increased with age, and immune individuals had a higher median breadth score compared to the few

adults who developed a clinical episode of malaria over the three-year follow-up period. Interestingly, all the adults evaluated in this study had a response to at least one of the novel antigens. This is in contrast to responses in children in which breadth scores of zero have been reported albeit to a different panel of merozoite antigens [496, 510, 515]. Taken together, these results are encouraging and warrant further evaluation in classic cohort studies involving children and adults from regions of varying transmission intensities. Similarly, these targets should be evaluated further using samples that are available in-house from controlled human malaria infection studies to identify responses associated with protection [151, 152].

#### ***Novel antigens associated with lower asymptomatic parasite densities***

A trend towards lower parasite densities measured at the cross-sectional surveys was observed in individuals who had a stable response to a majority of novel antigens (6/11 antigens; Table 5.7). This was significant for PF3D7\_1308000 and the trend was evident for three other novel antigens that were also associated with protection from clinical episodes of malaria: PF3D7\_0629500, PF3D7\_1025300 and PF3D7\_1401600(PHISTb). Additional studies are required to elucidate the role of these antigens in controlling parasite densities in both children and adults.

#### ***Unanswered questions***

While I demonstrate that antibody responses to a subset of these novel antigens appear to be associated with protection from malaria, the mechanisms by which they mediate their protection have yet to be elucidated. Antigen-specific functional responses monitored in either naturally exposed individuals or immunized animal models would provide valuable information to guide selection of new vaccine candidates. This would include evaluating the degree to which responses to these targets are effective against diverse parasite isolates. As antigenic polymorphisms have been a major obstacle in vaccine efficacy for antigens such as AMA1 and MSP-2, focusing on targets of limited polymorphisms is thought to be one way of overcoming this hurdle. Interestingly, the majority of novel targets evaluated (9/11: Figure 5.6) had a lower non-synonymous/synonymous SNP ratio compared to the

known merozoite antigens evaluated suggesting that they contained limited polymorphisms and were potentially attractive vaccine candidates.

Little is known about the temporal dynamics of humoral responses to malarial antigens in adults with the exception of MSP1 [491, 492] and MSP2 [491]. I show that antibody responses to a panel of known and novel antigens were stable over time in adults living in a hyperendemic village of Tanzania where malaria transmission was sustained throughout the study period. However, the longevity and stability of these responses in the absence of exposure or in the context of decreasing transmission intensity has yet to be elucidated. Recently, clinical episodes of malaria have been reported increasingly in adults living in endemic areas such as Senegal[516, 517] and Gabon[518], following reductions in malaria transmission due to introduction of control measures such as insecticide treated bednets[10, 516, 517]. The ideal effective vaccine should elicit a long-lived response following vaccination that would sustain protection even in the absence of exposure.

### ***Study limitations***

The study had several limitations, largely due to the nature of the samples and data that were available. A major limitation was that the numbers of clinical episodes in the cohort of adults were few, limiting the potential to detect true effects in the association studies. Secondly, temperatures were not routinely recorded during the cross-sectional surveys and at the health centre, although fevers were reported (personal communication, A. Farnert). As such, the definition of a clinical episode of malaria is less stringent than that typically used in immuno-epidemiology studies. However, this is mitigated by the fact that our study was focused on adults widely known to be clinically immune. In addition, data on identification of pregnant individuals within the cohort in the 1993 and 1994 survey is not available. This leaves open the possibility that a proportion of the few clinical cases observed at these time points were attributable to pregnancy-associated malaria. Fortunately, the numbers are small and would have minimal impact on the study. Lastly, due to the

time constraints of a PhD, I analysed antibody responses to only the 3D7 allele (originally from West Africa) of each antigen. Using this sequence may underestimate responses to more regionally representative sequences from East Africa. This also limited my ability to evaluate the effect of antigenic diversity on humoral responses and immunity.

### ***Epidemiology of malaria in Nyamisati, Tanzania***

Little is known on the contribution of antigen-specific antibody responses on the clinical immunity observed in adults living in a region of high transmission intensity. I therefore analysed the available epidemiological data from a cohort of longitudinally monitored individuals living in rural Tanzania so as to select a population of adults whose antibody responses would be analysed. Based on the *PfPR*<sub>2-10</sub>, the Nyamisati village would be classified as an area with a hyperendemic malaria transmission[511]. This classification would have remained unchanged had the parasitaemia been detected by microscopy or the more sensitive PCR method. Although microscopy is known to underestimate the parasite prevalence [519], the magnitude of this difference was particularly striking in the older age groups as has been reported before[519]. This has important implications for control programs aimed at reducing transmission, that rely on microscopy to monitor progress as the undetected low-density parasitaemia in adults may sustain larger than expected reservoirs for malaria transmission.

The epidemiology of malaria in Nyamisati was similar to that described in other areas that are subject to a high malaria transmission intensity [11, 76, 119]. The majority (over 90%) of adults were clinically immune with a large proportion (over 40%) carrying asymptomatic infections, consistent with the observation that sterile immunity is not achieved. Parasite prevalence rose in the youngest children, reaching a peak in older children and gradually declined with age in adulthood. Parasite densities also declined with age, in keeping with observations from other studies showing that adults living in a malaria-endemic area have the ability to control parasite densities upon *P. falciparum* infection [119] [487, 488]. Few

clinical episodes of malaria were observed in individuals older than 14 years of age, in agreement with reports of the acquisition of anti-disease immunity in older age-groups [520]. This clinical immunity appears to be stable as the prevalence of clinical malaria remained low over the three years of observation. Multiple clinical episodes of malaria were rare in adults and the few that occurred did so predominantly in pregnant women who are known to be susceptible [16].

Interestingly, I observed that the few adults treated at the health centre had three-fold lower median parasite densities (3500 p/ $\mu$ l) compared to the under 15 years olds treated at the health centre (10666 p/ $\mu$ l). Similar findings were reported from a treatment re-infection study in adults living in a holo-endemic village in Ghana where symptomatic adults had mean parasite densities of less than 500 parasites/ $\mu$ l [521], although a comparison with symptomatic children was not provided. Similarly in Mali, although fewer episodes of malaria were observed in adults (15-25 years), in these individuals, the median parasite densities were three fold lower than those reported in children (4-6 years)[487]. This would suggest that protection against clinical episodes of malaria in adults is at least in part mediated through mechanisms that control parasite density. On the other hand, one could argue that among susceptible adults, the threshold of parasitemia that results in a clinical episode is significantly lower than that observed in children. However, given the observation that asymptomatic parasite densities are also lower in adults compared to children, the most plausible explanation is that adults are better able to suppress parasite densities than children.

### ***In conclusion***

I present data identifying six novel antigens with limited polymorphisms whose humoral responses appear to be associated with protection from malaria in adults. These responses appear to be stable in adults over the three years. In chapter 6, I investigate the protective mechanisms by which antibodies to these novel antigens may function.

## CHAPTER 6

### **Antibody mediated effector functions against merozoites in immune adults**

#### **6.0. Introduction**

Identifying the targets of protective antibodies and the mechanisms by which they mediate protection is important for vaccine development. While naturally acquired *P. falciparum* antibodies have been shown to be effective by limiting blood stage replication and reducing parasite densities [127], little is known about the specific mechanisms by which they achieve this against the merozoite stage of the life-cycle. *In vitro* assays that are aimed at identifying the vaccine induced and naturally acquired immune correlates of protection to malaria continue to be developed and validated [136]. To date, most have focused on two proposed mechanisms against the merozoite stage of the life-cycle: 1) direct neutralization and thereby inhibiting merozoite invasion of erythrocytes and subsequently parasite growth or 2) interaction with leucocytes releasing effector functions that leads to parasite death.

The *in vitro* growth inhibitory assay (GIA), which measures the ability of antibodies alone to inhibit *P. falciparum* growth, has been the most widely used method for evaluating naturally-acquired and vaccine induced antibody effector functions against *P. falciparum* blood stages. For example, the GIA has been used to demonstrate the antigen specific activity for the potential merozoite vaccine targets MSP1-19 [522], RH4 [282] and RH5 [523] using affinity purified immunoglobulins from pools of malaria immune adults. In addition, it has been used to evaluate whole serum samples from children in prospective cohort studies albeit with conflicting results [136]. A second assay developed in the early 1990s demonstrated that naturally-acquired [150] and vaccine induced antibodies interacted with monocytes via their FcγII receptors releasing a soluble factor that resulted in parasite growth inhibition. This antibody-dependent cellular inhibition (ADCI) assay was subsequently used to demonstrate parasite inhibitory activity in sera from vaccinees that received the MSP2-C1 (containing the 3D7 and FC27 alleles of MSP2)[524]



and GMZ2 (containing the N-terminal of GLURP and the C-terminal of MSP3) vaccines[525]. Little is about the contribution of ADCI to naturally acquired malaria immunity in both children and adults. More recently, several new *in vitro* assays have been developed to elucidate the interaction between antibodies and monocytes/macrophages, neutrophils and soluble complement factors to trigger anti-merozoite (and other blood stages) responses. These assays included: 1) the direct phagocytosis of merozoites by monocytes opsonized by antibodies [139, 526] 2) interaction of merozoite specific antibodies with neutrophils resulting in the release of reactive oxygen species (ADRB) [140, 141, 527] and 3) the ability of antibodies to fix complement soluble factors onto the surface of merozoites resulting in cell lysis [138]. Similar assays had previously evaluated these immune effector mechanisms with more readily available stages of the parasite life cycle such as the opsonic and non-opsonic phagocytosis of *P. falciparum* infected erythrocytes[148], mature schizonts[528] and mature gametocytes[529]. However, the recently developed ability to purify viable merozoites [370] has facilitated the evaluation of these effector mechanisms specifically in anti-merozoite immunity for the first time.

Each of these assays has been evaluated in prospective cohort studies in children to elucidate their contribution to naturally acquired immunity. For example, opsonic phagocytosis of merozoites has been associated with protection from clinical episode and high parasite densities in Kenyan [139] and Papua New Guinea children [149, 530]. Similarly, opsonisation of merozoites with antibodies that result in neutrophil respiratory burst has been associated with clinical protection from malaria in Senegalese children [140] and protection from severe malaria in Kenyan infants when analysed in combination with growth inhibitory antibodies [515, 531]. Lastly, pools of antibodies from Kenyan and Papua New Guinean adults were shown to be able to fix complement factor C1q onto the surface of free merozoites leading to the formation of a membrane attack complex and presumably lysis of the merozoites. This ability was subsequently shown to be associated with protection from high-density parasitaemia and clinical episodes in Papua New Guinea children

[138]. It is notable that all of these studies to date have focused on responses in children, and consequently, the contribution of these effector functions to mediating immunity in adults has not been evaluated. In addition, little is currently known about whether the same individuals are able to produce multiple effector functions, or how stable or long-lived these functions are. Finally, the specific merozoite targets that elicit these protective functional responses are not currently known, and is critical for vaccine target prioritization.

## **6.1. Rationale**

With the exception of the use of adult sera in the development and validation of these *in vitro* assays, little is known about the role of these new protective mechanisms in immune adults. For example, pools of sera collected from adults living in malaria endemic regions of Kenya, Papua New Guinea and Senegal were used in the optimization of the opsonic phagocytosis [139], C1q-fixing [138] and the ADRB [140] assays respectively, but nothing is known about the prevalence, levels and contribution to immunity of each of these mechanisms, either on their own or in combination. I reasoned therefore that a cohort of longitudinally monitored adults living in a highly endemic area of Tanzania was an ideal platform for the evaluation of potential mechanisms of protective immunity. In particular, I measured three immune effector mechanisms namely 1) opsonic phagocytosis of merozoites, 2) antibody-dependent respiratory burst and 3) antibody-dependent recruitment of C1q onto merozoites in serum from adults collected at three consecutive time points. I evaluated: 1) the contribution of each of these mechanisms to immunity to clinical episodes, 2) the stability of these responses over a three year period, 3) the breadth of effector functions in adults and 4) correlations with antibody responses to the new antigens described in the preceding chapters.

## 6.2. Overall Objectives

To use sera from a cohort of longitudinally monitored adults to study antibody mediated effector responses, test for association with protective immunity and test the role of specific antigens in these functional responses.

## 6.3. Specific Objectives

- Describe antibody mediated effector mechanisms associated with protective immunity in adults.
- Identify merozoite antigens associated with protective antibody mediated effector functions.

## 6.4. Methods

### 6.4.1. Isolation of *P. falciparum* merozoite and RBC membrane enclosed merozoites

I obtained merozoites and RBC membrane enclosed merozoites from *P. falciparum* 3D7 or D10 strains using the same protocol described in section 2.4.1 with the following modifications. Segmented schizonts (approximately 44hpi) were incubated with 10µM of E64, (trans-Epoxy succinyl-L-leucylamido (4-guanidino) butane), for 6-8 hours. E64 is a protease inhibitor, which prevents merozoite egress from schizonts[370]. Treated schizonts were pelleted by centrifugation and resuspended in fresh media, counted using a haematocytometer and stored at -80°C as PEMS. To obtain merozoites, resuspended schizonts were filtered through a 1.2µm Acrodisc filter to release merozoites. Pelleted merozoites were resuspended in 1ml of RPMI media and quantified by flow-cytometry and used immediately in subsequent assays. Merozoites were stained with 100µg/ml of ethidium bromide for 10 minutes and incubated with Count-bright absolute counting Beads (Invitrogen) at a 1:10 dilution before data acquisition and quantification by flow cytometry. The formulae below was used for quantification:

$$\text{Merozoite concentration} = \frac{(\text{count of merozoite (EtBr+ population)})}{(\text{Count of beads})} \times \frac{(\text{volume of beads})}{(\text{volume of merozoites})} \times (\text{Concentration of beads/ml})$$

#### **6.4.2. Antibody-mediated opsonisation and phagocytosis of *P. falciparum* merozoites assay (OPA)**

An optimised assay was used to measure the ability of sera from adults to opsonize merozoites for phagocytosis by monocytes[139]. Briefly, THP-1, a human monocytic leukemic cell line[532], was grown at 37°C in 5% carbon dioxide in RPMI media that was supplemented with 10mM HEPES, 1% penicillin streptomycin, 2mM L-glutamine and 10% fetal calf serum. The cells were maintained at a total density of between  $10^5$  and  $10^6$  prior to phagocytic assays. 30µl of merozoites/well at a concentration of  $5 \times 10^7$  merozoites/ml were incubated with individual sera at a 1:2500 dilution in a 96-well U bottom plate pre-coated with 1% casein for 1 hour at room temperature. Wells were washed thrice using RPMI media and merozoites resuspended in THP-1 culture media. Phagocytosis of opsonized merozoites was performed by incubating 100µl of THP-1 cells at a cell density of  $5 \times 10^5$ /ml with 50µl of opsonized merozoites for 10 minutes at 37°C. Phagocytosis was stopped by centrifugation at 4°C and cells washed once with FACS buffer (1% Fetal bovine serum/0.05% sodium azide in PBS) and fixed with 2% ice-cold paraformaldehyde before data acquisition by flow cytometry, where a minimum of 4000 events was collected for each sample. The population of THP-1 cells that stained ethidium bromide positive indicated phagocytosis of *P. falciparum* merozoites. In each plate, several control samples were employed that included: a) unopsonised THP1 cells where no sera was added, positive control samples where b) a pool of hyper-immune sera (PHIS), c) purified immunoglobulins from immune adults (MIG) and d) negative control sera from non-malaria exposed adults living in Sweden. The relative phagocytosis index (RPI) for each serum sample was calculated as follows using reading obtained with PHIS sera as the benchmark:

$$\text{RPI} = \frac{(\% \text{ EtBr+ population of THP1 cells opsonized with sample sera})}{(\% \text{ EtBr+ population of THP1 cells opsonized with PHIS sera})} \times 100$$

All serum samples were assayed in duplicate and the mean RPI of duplicate readings calculated. The prevalence of antibodies mediating phagocytosis was

calculated using the mean RPI plus 3 standard deviation values of non-malaria exposed sera from Swedish adults (N=14) as the cut-off point

#### ***6.4.3. Antibody mediated C1q fixation by *P. falciparum* merozoites***

I used a recently developed and published ELISA-based C1q fixation assay[138] to measure the ability of merozoites to recruit the complement factor C1q. To optimize PEMS coating concentration to use in the C1q fixation assay, PEMS were serially diluted to determine and tested against a pool of immune sera from malaria-exposed adults and non-exposed adults as a negative control. Thereafter, 100µl of PEMS at a concentration of  $10 \times 10^5$  PEMS/ml was used for all serum samples. PEMS were coated on a 96-well flat-bottomed immulon 4HBX plates overnight at 4°C. Wells were then washed thrice with PBS and blocked with 200µl of 10% skimmed milk for 2 hours at 37°C. This was followed by incubation with 50µl of sera at a 1:200 dilution in 5% skimmed milk for 2 hours at room temperature. Wells were washed and incubated with recombinant human C1q (Calbiochem) at a 10µg/ml concentration for 30 minutes at room temperature. This was followed by incubation with 50µl of rabbit anti-C1q antibodies at a 1:2000 dilution for 1 hour at room temperature. Wells were washed thrice as before and incubated with 50µl of anti-rabbit conjugated to horseradish peroxidase at a 1:2000 dilution for 1 hour at room temperature. Wells were then washed and incubated with 100µl of O-phenylenediamine (OPD) for 15 minutes at room temperature and reaction stopped by adding 25µl of 2M H<sub>2</sub>SO<sub>4</sub> and the absorbance read at 492nm using the Biotek Synergy 4 plate reader and Gen5 acquisition software. All serum samples were assayed in duplicate.

#### ***6.4.4. Antibody-dependent respiratory burst (ADRB)***

I measured ADRB activity in serum samples using an optimised assay that had been set up in-house[515, 531]. The serum samples were measured in duplicate using freshly isolated neutrophils obtained from two healthy adults.

#### *6.4.4.1. Neutrophil isolation from healthy donors*

Forty (40) ml of whole blood was collected from healthy individual donors in heparin vacutainer tubes and mixed with an equal volume of Hanks buffered salt solution (HBSS; Sigma Aldrich) with  $\text{NaHCO}_3$  and layered over ficoll (Ficoll-paque Plus; Sigma Aldrich) and centrifuged for 15 minutes to separate blood components. Serum, peripheral blood mononuclear cells and ficoll layers were carefully removed and the remaining red blood cells that contained granulocytes resuspended in HBSS buffer. This was then gently mixed with 3% dextran solution at a 1:3 dilution and incubated at room temperature for 1 hour in the dark. The supernatant that contained neutrophils was carefully collected and pelleted and the red blood cell contaminants lysed by sequential treatment with ice-cold 0.2% and 1.6% NaCl solutions respectively. Pelleted neutrophils were resuspended in sterile-filtered polymorphonuclear cell buffer (PMN buffer: 0.1% bovine serum albumin, 1% D-(+)-Glucose, HBSS) and counted using a hemocytometer observed under x400 magnification using the formulae below, before readjustment to a concentration of  $10 \times 10^6$  neutrophils/ml.

Neutrophil concentration/ml = (count of neutrophils in 25 squares) X (dilution factor) X  $(10^4)$  = cells/ml

#### *6.4.4.2. ADRB assay*

PEMS stored at  $-80^\circ\text{C}$  were serially diluted to determine the coating concentration for subsequent assays and tested against a pool of immune sera from malaria-exposed adults and non-exposed adults as a negative control. After optimization, 100 $\mu\text{l}$  of RBC membrane ec at concentration of  $15 \times 10^5$  PEMS/ml were coated on a 96-well flat-bottomed white polystyrene fluorescence maxisorp plates overnight at room temperature in the dark. Wells were washed 3 times with PBS and blocked with 200 $\mu\text{l}$  of 10% casein in PBS for 1 hour at room temperature. Following a second wash step with PBS, 50 $\mu\text{l}$  of sera diluted at 1:50 in PBS was added for 1 hour at  $37^\circ\text{C}$  before an additional wash with PBS and the addition of 50 $\mu\text{l}$  of neutrophils at a  $10 \times 10^6$  neutrophils/ml concentration per well. 50 $\mu\text{l}$  of isoluminol at 0.04mg/ml concentration was rapidly added to each well and luminescence

measured in each well every 2 minutes over a duration of 60 minutes using the Biotek Synergy 4 plate reader and Gen5 acquisition software. The maximal RLU measured in each serum was obtained. All serum samples were assayed in duplicate using neutrophils obtained from two different healthy donors. The relative light units (RLU) for each serum sample was calculated as follows using responses obtained from the MIG positive control included on each plate as the benchmark:

$$\text{RLU} = \frac{\text{Maximum light units in the sample}}{\text{Mean maximum light units of 4 MIG samples included in each plate}}$$

Mean maximum light units of 4 MIG samples included in each plate

## 6.5. Results

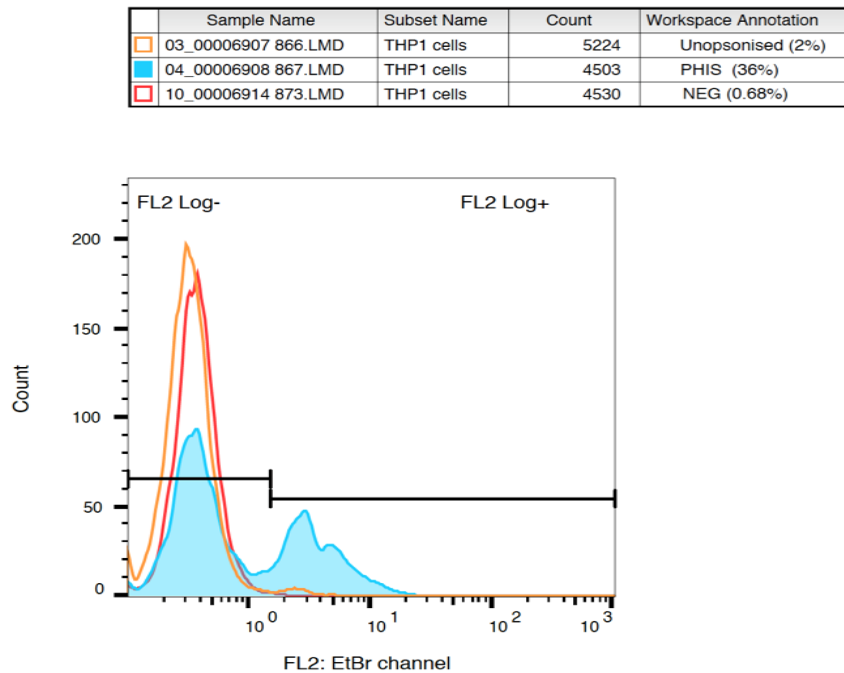
Serum samples collected from the same 77 individuals in three consecutive cross-sectional surveys were available for functional assays resulting in a total of 231 samples. The samples were used to measure the ability to opsonize and phagocytosis of merozoites, interact with neutrophils for ADRB activity and antibody-dependent recruitment of C1q onto merozoites.

### 6.5.1. Opsonic-phagocytosis of merozoites by sera from adults

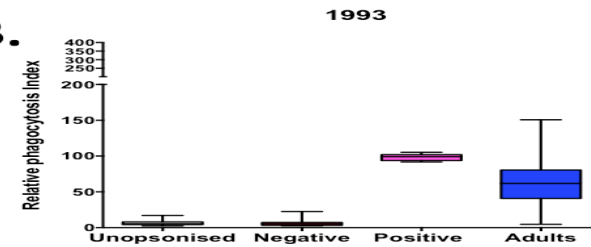
Serum samples were tested for the presence of antibodies that mediate opsonic phagocytosis of *P. falciparum* merozoites (OPA). The relative phagocytosis index was calculated for each adult sample, using hyper-immune sera as the benchmark sample as described in section 6.4.2. As shown in Figure 6.1A, merozoites opsonized with hyper-immune sera (PHIS) were phagocytosed to a greater extent compared to non-malaria exposed sera or unopsonised THP-1 cells evidenced by a higher proportion of ethidium positive THP1 population (36% in PHIS compared to 0.68% and 2% in negative and unopsonised samples respectively (Figure 6.1A)). The relative phagocytosis indices computed for individuals varied from as low as 1.7 to as high as 321.7. As shown in Figure 6.1B, C and D, the median (min-max) RPI value of Nyamisati adult sera was 61.8 (4.6-150.6), 68.3 (1.7-321.7) and 56.3 (8.2-196.4) using samples collected at the 1993, 1994 and 1995 surveys respectively. The prevalence of antibodies mediating phagocytosis was determined based on a cut-off generated by calculating the mean plus 3 standard deviation RPI of sera from non-exposed Swedish adults. Opsonizing antibodies were detectable in the majority of adults tested resulting in prevalence's of 90.9%, 93.5% and 96.1% in the 1993, 1994 and 1995 cross-sectional surveys respectively. As the serum samples were not heat-inactivated to remove the effect of complement, the above assay would measure opsonisation by antibodies alone, complement alone or both these factors if present in the serum sample.



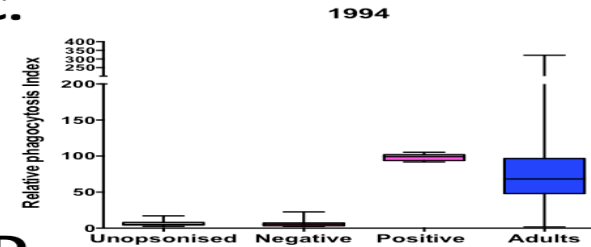
## A. PLATE 1A



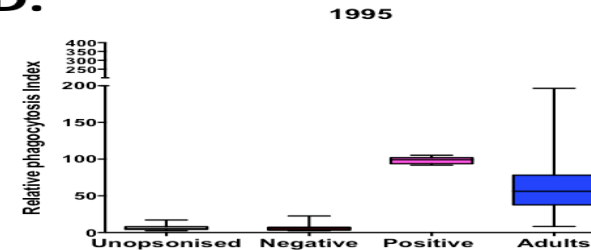
## B.



## C.



## D.



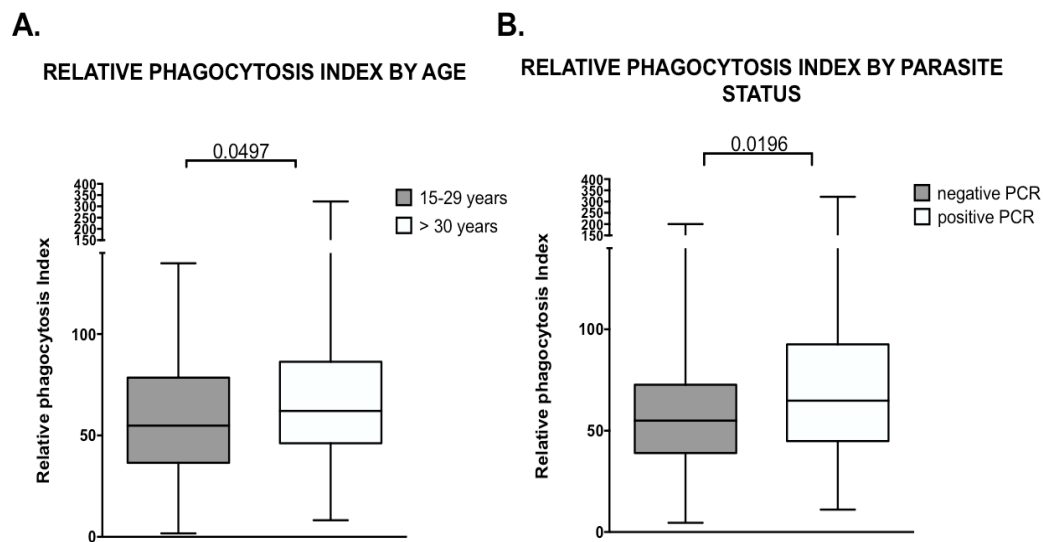
**Figure 6.1: Antibodies mediating phagocytosis of *P. falciparum* merozoites by opsonisation and interaction with THP-1 cells in adults of the Nyamisati cohort.** (A) A flow cytometry histogram overlay contrasting phagocytosis in THP-1 monocytic cell lines in freshly isolated merozoites opsonized with PHIS (blue), non-malaria exposed sera (red) and unopsonised THP-1 cells (orange). A comparison of the relative phagocytosis index (RPI) calculated for each individual samples collected at the 1993 (B), 1994 (C) and 1995 (D) cross-sectional survey with indices obtained in control serum samples. The RPI values were higher in adults (blue box and whisker plots) compared to the negative control that was serum from non-exposed adults from Sweden. The box shows the median and interquartile RPI for the 77 individuals/survey while the whiskers show the minimum and maximum measured.

RPI

measured.

### 6.5.1.0. Association between opsonic-phagocytosis, age and asymptomatic parasitaemia in adults

I measured OPA in adults whose ages ranged from 15 years to 80 years. I categorized the individuals into two age categories, those between 15 to 29 years and those 30 years and older at each survey. I then pooled the data from individuals who fell within these age categories and compared their RPI. As shown in Figure 6.2A, the median relative phagocytosis indices were significantly higher in the older age group: median RPI between 15-29 years versus > 30 years was 54.81 versus 62.07,  $p$ -value=0.0497. A weak positive correlation of an  $R_{\text{pearson}}=0.1251$ ,  $p$ -value=0.058 was observed between age and the phagocytosis indices. A similar trend was observed when data from each cross-sectional survey was analysed independently as shown in Appendix 6.1, Figure 1A.



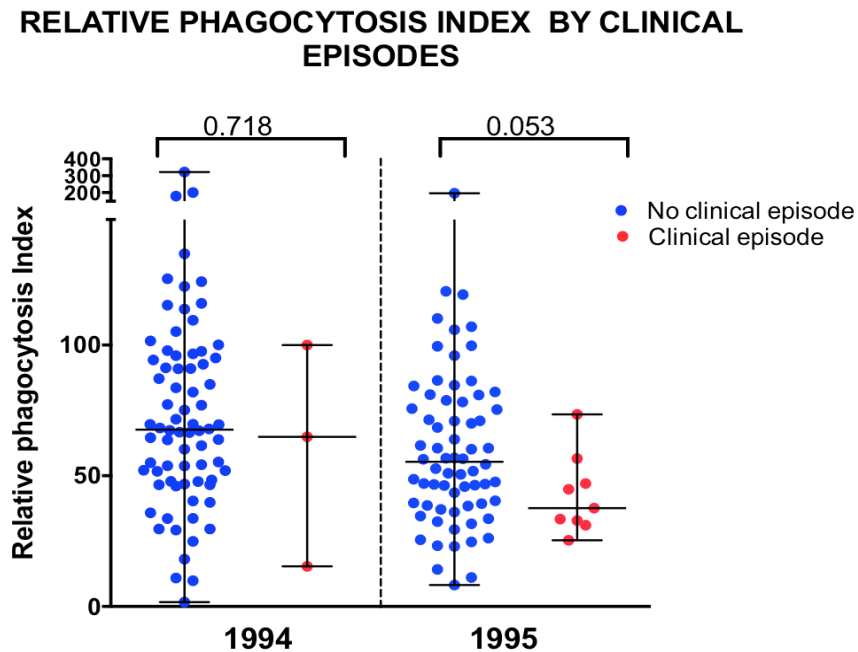
**Figure 6.2: Relative phagocytosis indices in adults grouped by age and parasite status.** (A) A comparison of the median phagocytosis indices in adults 30 years and older (white box and whisker plots) compared to 15-29 year olds. (B) Median phagocytosis indices in adults with asymptomatic parasitaemia (as measured by qPCR of the 18S gene) compared to those who were parasite negative (black box and whisker plots). Data presented is pooled from the three cross-sectional surveys.

Similarly, the phagocytosis indices were compared in individuals who were parasite positive or negative at any of the three cross-sectional surveys as measured by

qPCR detection of the 18S ribosomal RNA gene (Figure 6.2B). These indices were elevated in PCR positive individuals, which was statistically significant: median RPI in parasite negative adults compared to parasite positive individuals were 55.03 versus 64.87, p-value=0.0196. A similar trend was observed when data from each cross-sectional survey was analysed independently as shown in Appendix 6.1, Figure 1B.

#### *6.5.1.1. Association between opsonic-phagocytosis and clinical immunity in adults*

All participants in the annual cross-sectional surveys were monitored for clinical episodes of malaria throughout the year at the single health centre that serves the entire village. A malaria episode was defined as a fever (axillary temperature of  $> 37.5^{\circ}\text{C}$ ), detectable parasites by microscopy and the absence of clinical findings related to other infections. Of the 77 individuals, none of them experienced a clinical episode in the 1993 follow-up period, while 3 (3.9%) and 9 (11.7%) individuals experienced a clinical episode in the 1994 and 1995 follow-up periods respectively. I compared the phagocytosis indices in individuals who did and did not experience a clinical episode of malaria. As shown in Figure 6.3, although the numbers are small, in the 1994 follow-up period the median phagocytosis indices at the cross-sectional survey were lower in individuals who experienced a clinical episode compared to those who did not although this was not statistically significant (Mann-Whitneys U test: 64.87 versus 67.65; p-value=0.718). Similarly, in the 1995 follow-up period, median indices were lower in individuals who experienced a clinical episode compared to those who did not (37.51 versus 55.36; p-value=0.053), which was borderline significant.

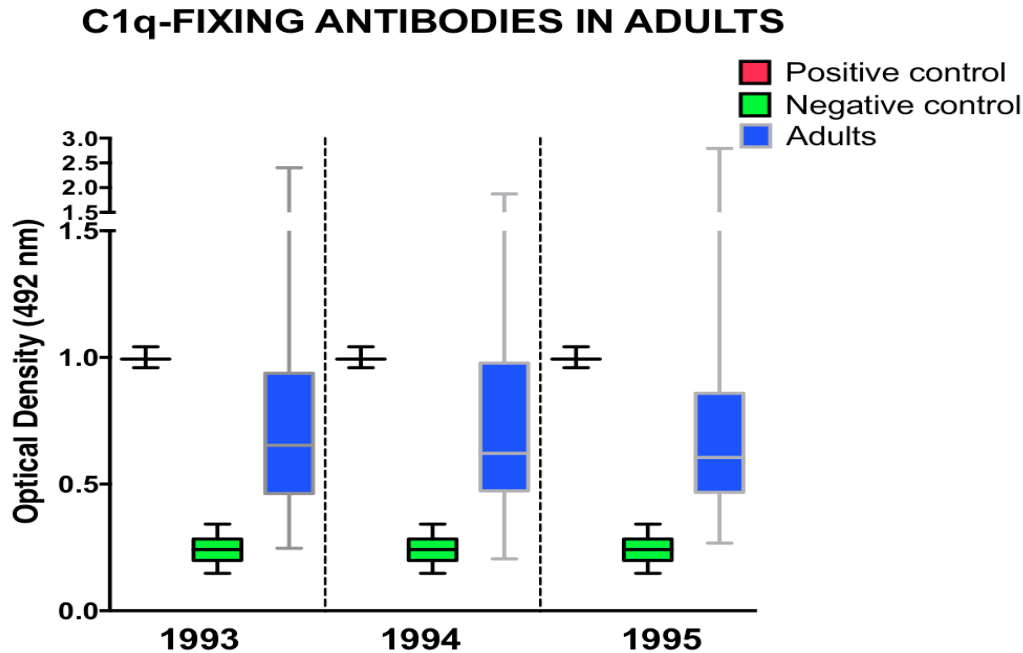


**Figure 6.3: Relative phagocytosis indices in adults who experienced a clinical episode of malaria compared to those who did not in the 1994 and 1995 follow-up period.** Blue circles represent adults who did not experience a clinical episode while red circles represent those who did.

### 6.5.2. C1q-fixing antibodies in sera from adults

I used an established protocol to measure C1q-fixing antibodies in sera from adults in the Nyamisati cohort [138]. I optimised the coating concentration of PEMS for subsequent assays by testing a serial dilution of merozoites against a pool of hyper-immune sera and non-exposed sera as a negative control. As shown in Appendix 6.1, Figure 2, I selected a coating concentration of  $10 \times 10^5$  PEMS/ml for all subsequent assays, as this was within the linear range and showed differential activity between the positive and the negative control samples. As shown in Figure 6.4, the median optical density (min-max) measured for C1q-fixing antibodies in 77 adults were 0.65(0.25-2.40), 0.62(0.21-1.87) and 0.61(0.27-2.79) in samples collected at the 1993, 1994 and 1995 surveys respectively. These levels were higher than responses measured in serum from non-exposed individuals. The prevalence of C1q-fixing antibodies was determined by calculating the proportion of individuals with responses above the mean plus 3 standard deviation of 12 non-exposed serum

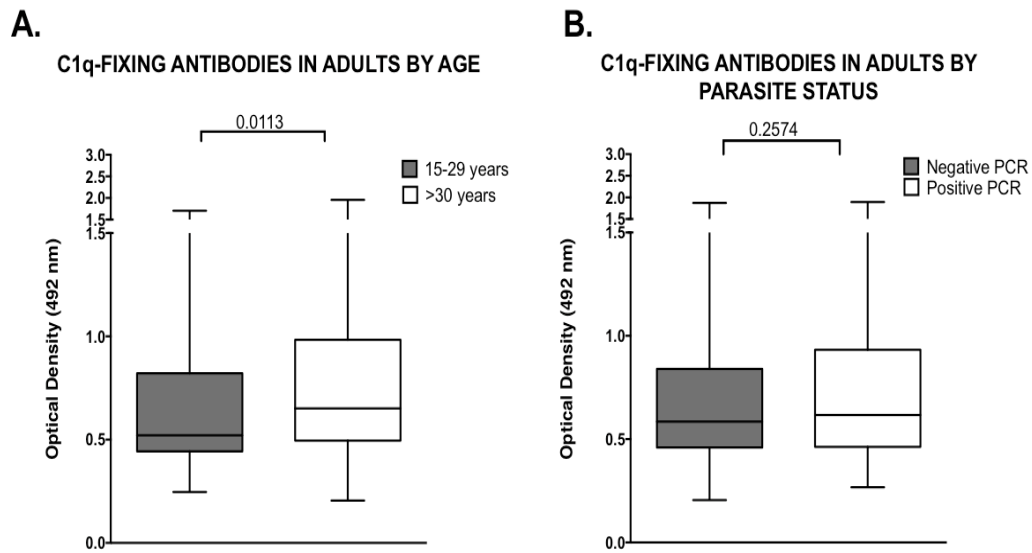
samples and were as follows: 59 (76.6%), 64 (83.1%) and 65 (84.4%) at the 1993, 1994 and 1995 surveys respectively.



**Figure 6.4: C1q-fixing antibodies in adults of the Nyamisati cohort.** C1q-fixing antibodies in sera samples from adults of the Nyamisati cohort collected at the 1993, 1994 and 1995 cross-sectional surveys. Green box and whisker plots represent OD values in serum samples from 12 non-exposed serum samples from Swedish adults. Blue box and whisker plots represent the median and minimum/maximum OD values in serum samples from adults at each cross-sectional survey.

#### 6.5.2.0. Association between C1q-fixing antibodies by age and parasites status

I compared the levels of C1q-fixing antibodies in adults by age and their parasite status determined at the cross-sectional surveys. As shown in Figure 6.5A, C1q-fixing antibody levels increased with age with the median antibody levels in 15-29 year olds compared to 30 year olds being 0.5210 versus 0.6515;  $p$ -value=0.0113. A positive correlation of an  $R_{\text{pearson}}=0.2989$ ,  $p$ -value=0.0001 was observed between age and the C1q-fixing antibody levels. Similarly, C1q-fixing antibodies were elevated in adults with asymptomatic parasitaemia (parasite positive) compared to those who were not, although this was not statistically significant (Figure 6.5B respectively). Similar trends were observed when data from each cross-sectional survey was analysed independently as shown in Appendix 6.1, Figure 3.



**Figure 6.5: C1q-fixing antibodies in adults of the Nyamisati cohort by age and parasite status.** (A) Median anti-C1q fixing antibodies were higher in adults 30 years (white box and whisker plots) and older compared to 15-29 year olds (black box and whisker plots). (B) Median anti-C1q fixing antibodies were higher in adults with asymptomatic parasitaemia (as measured by qPCR of the 18S gene) than in those who were parasite negative (black box and whisker plots). Data presented is pooled from the three cross-sectional surveys.

#### 6.5.2.1. Association between C1q-fixing antibodies and clinical immunity in adults

I compared the levels of C1q-fixing antibody in individuals who did or did not experience clinical episodes of malaria in the 1994 and 1995 follow-up periods. These were 3 and 9 individuals respectively. The median levels of C1q-fixing antibodies were significantly lower in the adults who experienced a clinical episode over the 1995 follow-up period. The median antibody levels were 0.608 versus 0.622;  $p$ -value=0.853 and 0.632 versus 0.449;  $p$ -value=0.013 in the 1994 and 1995 follow-up period as shown in Figure 6.6.

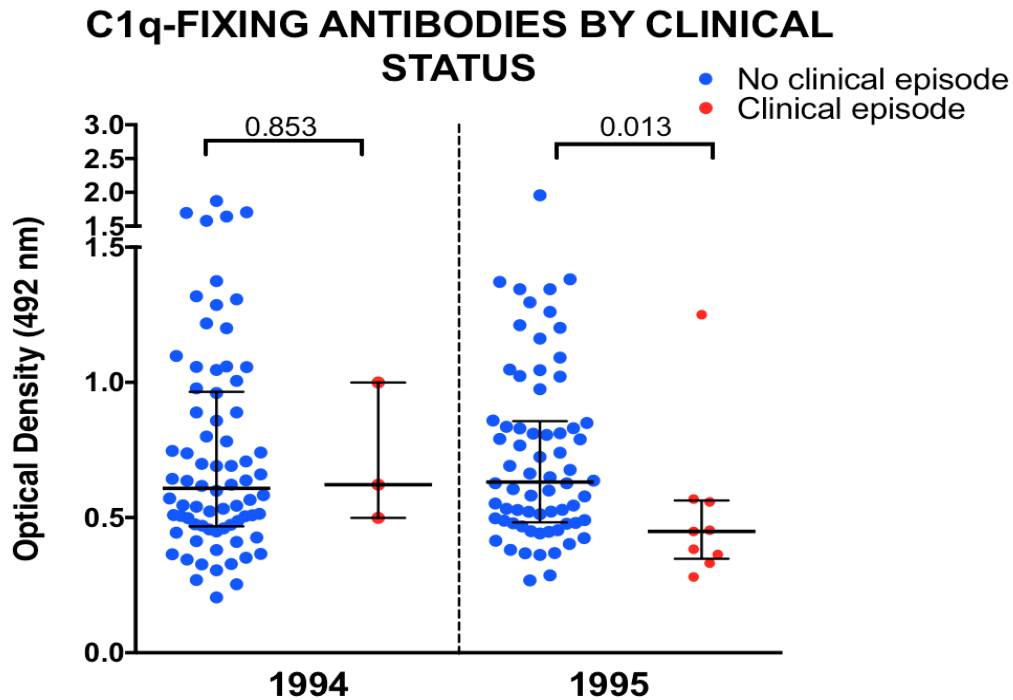
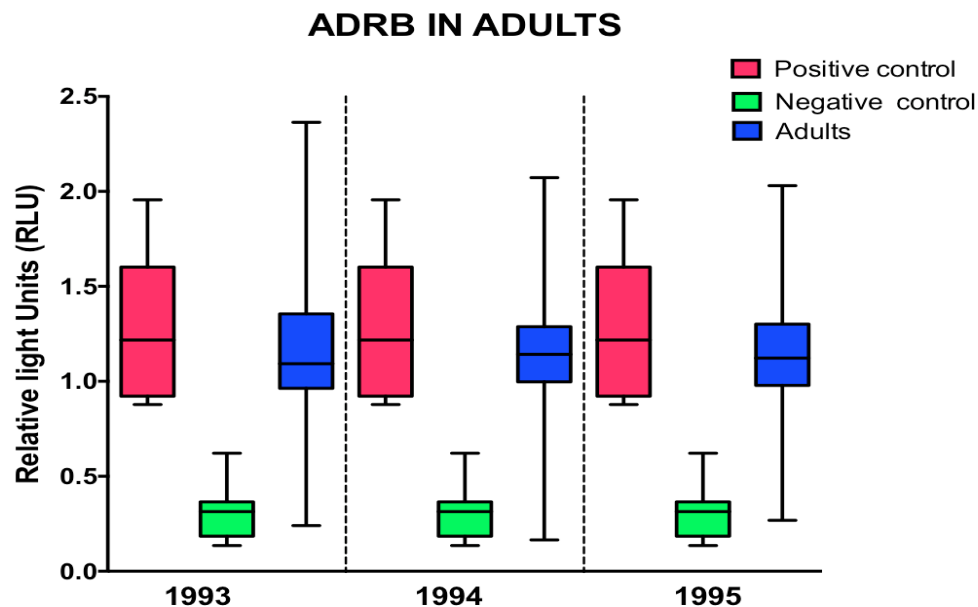


Figure 6.6: C1-q fixing antibodies in adults who experienced a clinical episode of malaria compared to those who did not in the 1994 and 1995 follow-up period. Blue circles represent adults who did not experience a clinical episode while red circles represent those who did.

### 6.5.3. Antibody-dependent respiratory bursts (ADRB) of merozoites by sera from adults

I used an optimised protocol to measure ADRB activity in serum sample from a cohort of immune adults. Prior to conducting the assays, a new batch of PEMS stored at  $-80^{\circ}\text{C}$  were serially diluted to optimize the coating concentration for subsequent assays using a pool of hyper-immune sera and non-exposed sera as a negative control. As shown in Appendix 6.1, Figure 4, I selected a coating concentration of  $10 \times 10^5$  PEMS/ml per well as this was within the linear range and showed differential activity levels between the positive and the negative control samples. The relative light units (RLU) were calculated for each sample as has been described in section 6.4.4.2. As expected, the median (min-max) RLU in the adult sera samples were higher than non-exposed adults and were 1.092 (0.241-2.364),

1.142 (0.165-2.072) and 1.122(0.268-2.031) for samples collected at the 1993, 1994 and 1995 cross-sectional surveys respectively (Figure 6.7). I used the mean plus 3 standard deviation RLU calculated from 15 non-exposed sera from Swedish adults as a cut-off for seropositivity. The prevalence of antibodies mediating ADRB activity was high at 76 (98.7), 74 (96.1) and 76 (98.7) adults at the 1993, 1994 and 1995 cross-sectional survey respectively.



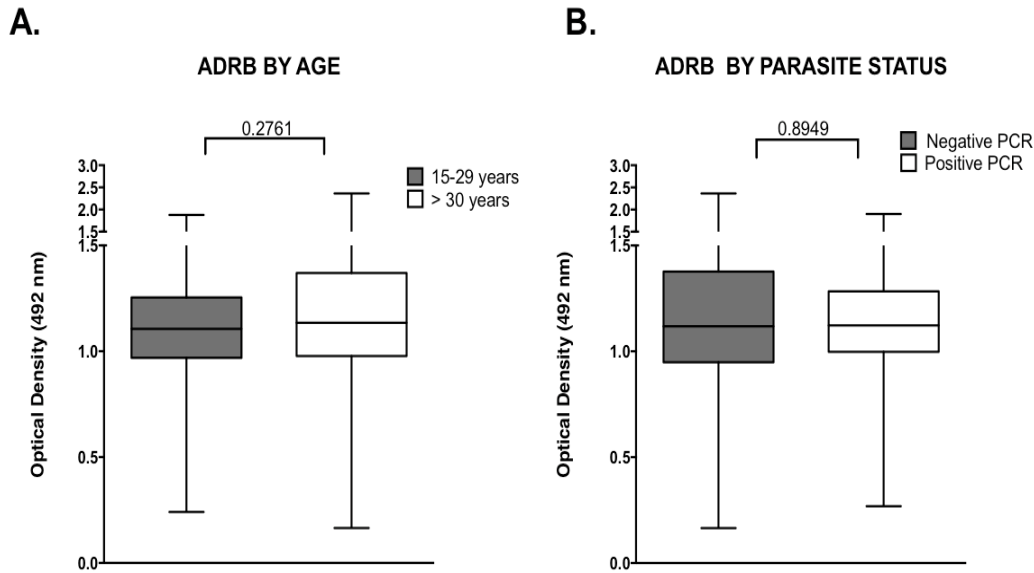
**Figure 6.7: ADRB activity in adults of the Nyamisati cohort.** ADRB in sera samples from adults of the Nyamisati cohort collected at the 1993, 1994 and 1995 cross-sectional surveys. Red box and whisker plots show the RLU values obtained in a pool of hyper immune sera samples from Kenyan adults (positive control), the green box and whisker plots represent RLU values in serum samples from 15 non-exposed serum samples from Swedish adults. Blue box and whisker plots represent RLU values in serum samples from adults.

#### 6.5.3.0. Association between ADRB activity by age and parasite status

I did not observe an increase in ADRB activity with age or parasite status as shown in Figure 6.8A and B respectively. Median RLU in 15-29 year olds compared to over 30 year olds were 1.106 versus 1.135,  $p$ -value=0.2761. A weak positive correlation of an  $R_{\text{pearson}}=0.1927$ ,  $p$ -value=0.0033 was observed between age and the adrb relative light units reported. Similarly, no trend with asymptomatic parasitemia was observed. ADRB activity in adults with asymptomatic parasitemia (parasite



positive) compared to negative individuals was not significantly different. Similar trends were observed when data from each cross-sectional survey was analysed independently as shown in Appendix 6.1, Figure 5.



**Figure 6.8: ADRB in adults of the Nyamisati cohort by age and parasite status.** (A) Median relative light units were not significantly different in adults 30 years and older (white box and whisker plots) compared to 15-29 year olds. (B) Median relative units were not significantly different in adults with asymptomatic parasitemia (as measured by qPCR of the 18S gene) than in those who were parasite negative (black box and whisker plots). Data presented is pooled from the three cross-sectional surveys.

#### 6.5.3.1. Association between ADRB and clinical immunity in adults

I compared the ADRB activity in individuals who did or did not experience clinical episodes of malaria. No differences were observed in the ADRB levels between these individuals. As shown in Figure 6.9, the median relative light units did not differ in those who experienced a clinical episode of malaria as follows; median RLU were 1.072 versus 1.163;  $p$ -value=0.2659 and 1.085 versus 1.137;  $p$ -value=0.178 in the 1994 and 1995 follow up periods respectively.

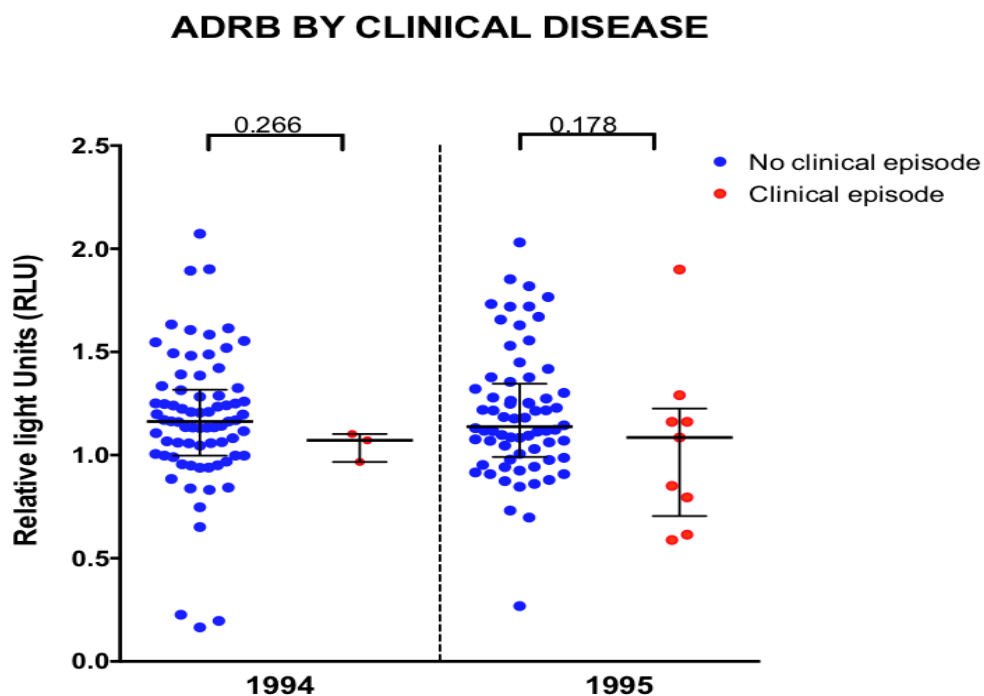


Figure 6.9: ADRB in adults who experienced a clinical episode in the 1994 and 1995 follow-up period.

#### 6.5.4. Stability and breadth of immune effector mechanisms in adults of the Nyamisati cohort

The prevalence of the three measured immune effector mechanisms were high in this cohort of adults ranging from 77% to 98% in the three cross-sectional surveys. As a result, I categorized these individuals as having either high or low levels of measured effector functions, if they had an above or below median levels measured at each respective survey. I then determined the stability of these levels over the three years. The majority of adults had stable ADRB and C1q-fixing antibodies over the three years. For instance, as shown in Table 6.1, 66% had stable ADRB levels measured, with 34% of them having above median ADRB responses and 32% below the median levels in all three cross-sectional surveys. Similarly, 62% of the adults had stable C1q-fixing antibody levels with 29% and 34% having above and below median levels in all three surveys respectively.

**Table 6.1: The stability of antibody effector responses in adults in the Nyamisati cohort.**

<b>Classification I</b>	<b>Opsonic-phagocytosis N (%)</b>	<b>C1q-fixing antibodies N (%)</b>	<b>ADRB levels N (%)</b>	<b>Classification II</b>
Above median levels in all three surveys	18 (23.4)	22 (28.6)	25 (32.5)	Stable high responders
Above median levels in 2/3 surveys	22 (28.6)	20 (26.0)	13 (16.9)	
Above median levels in 1/3 surveys	16 (20.8)	9 (11.7)	13 (16.9)	Non-responders
Below median levels in all three surveys	21 (27.3)	26 (33.8)	25 (32.5)	

The proportion of individuals classified as having above or below median levels of immune effector responses in each of the three surveys. In brackets is the proportion of adults who fell within the category.

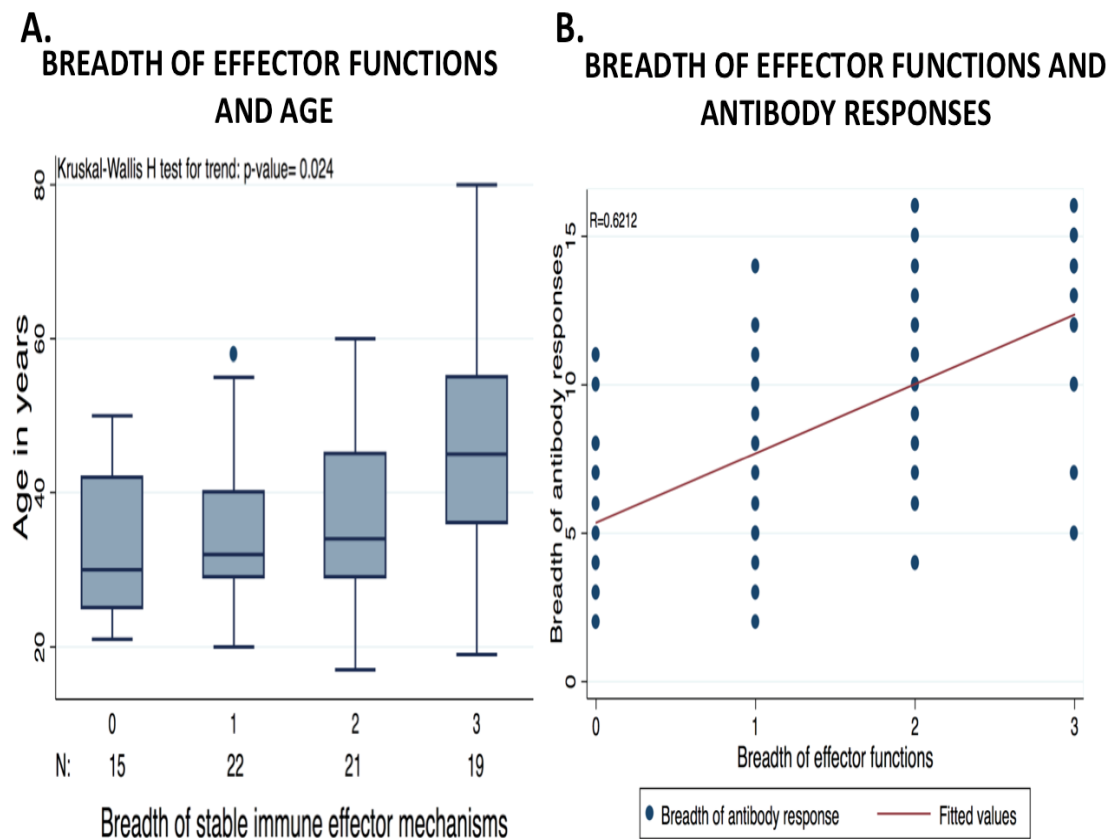
I then compared the parasite densities measured at each of the three cross-sectional surveys in individuals with stable responses versus non-responders. As shown in Table 6.1, stable high responders were defined as individuals with an antibody effector response above the median level in at least two of the three cross-sectional surveys and non-responders were individuals in whom the levels were below median levels at all three cross-sectional surveys or had an high-titre response in only one of the surveys. As shown in Table 6.2, no significant differences were observed in the parasite densities recorded in individuals who had a stable response to any of the 3 immune effector mechanisms measured.

**Table 6.2: The relationship between the stability of antibody effector responses and parasite densities (as determined by microscopy) in adults in the Nyamisati cohort.**

<b>Immune effector functions</b>	<b>Median log<sub>10</sub> parasite densities (95% confidence intervals)</b>		
	<b>Non responders</b>	<b>Stable responders</b>	<b>P-values</b>
<b>Opsonic-phagocytosis</b>	1.50(0.99-1.90)	1.50(0.90-2.20)	0.6589
<b>C1q-fixing antibodies</b>	1.50(0.96-2.35)	1.50(0.90-1.75)	0.3882
<b>ADRB levels</b>	1.20 (0.90-2.20)	1.51 (0.91-1.98)	0.5190
<b>Breadth scores</b>	0	3	

Lastly, I calculated the breadth of antibody effector responses by adding up the number of stable responders or non-responder in each assays. This resulted in individuals having one of the four possible breadth scores that ranged from 0-3. A

breadth score of 3 indicated that the individuals were categorized as a stable responder (Table 6.2) in all three functions. Using this classification, 15(19.5%), 22(28.6%), 21(27.3%) and 19(24.7%) had a breadth score of 0, 1, 2 and 3 respectively as shown in Figure 6.10. The breadth score increased with age as shown in Figure 6.10A, while individuals who experienced a clinical episode had a lower breadth score than immune individuals, although this was not statistically significant (median breadth score: 2 versus 1: p-value 0.1866). I also observed a strong positive correlation between the breadth of immune effector responses and the breadth of antibody responses to individual antigens (Figure 6.10B).



**Figure 6.10: The breadth of antibody effector function in adults.** The breadth increases with age (A) and with the breadth of antibody responses (B) in adults of the Nyamisati cohort. The breadth score of antibody responses to 16 antigens was correlated to the breadth of immune effector mechanisms measured in the same adults of the Nyamisati cohort.

#### ***6.5.5. Correlation between immune effector mechanisms and antibody levels to novel antigens***

Cumulatively, I measured antibody responses (total IgG) to 16 antigens that included 5 previously studied merozoite antigens and 11 novel antigens described in Chapter 5 of this thesis. I then determined the correlation between antibody levels to these antigens and the three immune effector mechanisms measured in the same individuals. As shown in Table 6.3, strong positive Pearson correlations (above 0.5: blue squares) were observed between MSP1-<sub>19</sub> and MSP-3 with C1q-fixing antibodies and MSP-2 with both ADRB and C1q-fixing antibodies. In the panel of 11 novel antigens, strong positive correlations were consistently observed in the three surveys between *Pf*PAT and both ADRB and C1q fixing antibodies. Similarly, a strong positive correlation was observed consistently between antibody levels to PF3D7\_073800.2 and C1q-fixing antibodies. Antibody levels to PF3D7\_1252300 were strongly correlated to C1q-fixing antibody levels in all surveys, while PF3D7\_1308000 were strongly correlated to ADRB and C1-fixing levels in at least one survey. PF3D7\_1401600(PHISTb) showed a strong correlation with C1q-fixing antibodies. Four antigens did not show a strong positive correlation to any of the three immune effector mechanisms measured.

**Table 6.3: Correlation between antigen-specific antibody responses and the levels of ARDB, C1q-fixing and opsonic-phagocytosis mediating antibodies**

Antigens	1993			1994			1995		
	ADRB	C1-q fixing antibodies	Opsonic phagocytosis	ADRB	C1-q fixing antibodies	Opsonic phagocytosis	ADRB	C1-q fixing antibodies	Opsonic phagocytosis
MSP1 <sub>-19</sub>	0.2301	0.5607	0.2892	0.2402	0.5418	0.1788	0.3027	0.4565	0.2591
AMA-1	0.3426	0.2293	0.1847	0.4589	0.2587	0.3254	0.2570	0.2403	0.1258
MSP-2	0.5532	0.6013	0.1298	0.4900	0.5970	0.1220	0.5637	0.5661	0.0862
MSP-3 (3D7 allele)	0.2149	0.4647	0.2746	0.3657	0.5646	0.0182	0.2767	0.5623	-0.0933
RH2b	0.0776	0.1432	0.2089	0.2381	0.1564	0.2433	0.2586	0.2870	0.0137
PF3D7_0206200( <i>Pf</i> PAT)	0.6236	0.5966	0.3154	0.5649	0.6655	0.0518	0.5156	0.5506	-0.1367
PF3D7_0629500 (SEG2)	0.3135	0.4225	0.1584	0.3427	0.4994	-0.0196	0.2338	0.5212	0.0284
PF3D7_073800.2	0.3624	0.6766	0.2409	0.4590	0.7006	0.1487	0.4669	0.6627	-0.0083
PF3D7_0823800	0.1812	0.2605	0.0839	0.3385	0.3778	0.0296	0.2633	0.3794	0.0077
PF3D7_0830500	0.3384	0.3911	0.0539	0.2734	0.3611	-0.0743	0.2719	0.4639	0.0914
PF3D7_0925900	0.2708	0.3116	0.2136	0.4033	0.4570	0.0969	0.4407	0.2829	-0.1430
PF3D7_1025300	0.2273	0.4342	0.1632	0.4369	0.4668	0.2141	0.3874	0.4829	0.0492
PF3D7_1252300	0.3180	0.5384	0.0253	0.3416	0.6322	0.0391	0.4009	0.6431	0.0802
PF3D7_1308000	0.4188	0.5225	0.2333	0.5416	0.6592	0.0786	0.4528	0.4543	-0.1017
PF3D7_1345100	0.3138	0.3990	-0.0239	0.3358	0.5152	-0.0185	0.3373	0.5307	-0.0554
PF3D7_1401600(PHISTb)	0.2763	0.5137	0.0325	0.2789	0.4756	0.1941	0.3364	0.6136	0.0864

Pearson correlation coefficients between antigen-specific antibody responses (optical density values) and ADRB activity (RLU), C1-fixing antibody levels (optical density values) and opsonic-phagocytosis (Relative phagocytosis indices). Squares highlighted in blue indicate a strong positive correlation coefficients > 0.5.

## 6.6. Discussion

### *Immune effector mechanisms in adults*

The main aim of this study was to describe immune effector mechanisms in a cohort of longitudinally monitored adults and test whether they are correlated with responses to the novel antigens identified in this thesis. A high prevalence (greater than 90%) of antibodies that opsonized merozoites resulting in phagocytosis and respiratory burst by neutrophils was observed in adults of the Nyamisati cohort. C1q-fixing antibodies were also detected in this population but to a lesser extent with a prevalence that ranged from 77-84% in the three surveys. The levels of these effector functions varied greatly between individuals and a trend of an increase with age and asymptomatic parasitaemia for opsonic-phagocytosis and C1-fixing antibody levels was observed. In contrast, age and parasite status did not seem to influence ADRB levels in this cohort of adults. These findings are similar to those from two cohorts in Senegal (age range in years: 3.4-76.9) in which ADRB levels were lower in the individuals with circulating parasites[140] compared to those who were parasite free. They differ however, from findings following experimental human infections with *P. falciparum* in malaria-exposed adults in which ADRB levels increased significantly following challenge[152]. It is worth noting that antibody levels to individual antigens in this cohort also did not vary with age (Chapter 5).

### *Immune effector mechanisms are associated with protection from clinical episodes of malaria*

Opsonic-phagocytosis and C1q-fixing antibodies appeared to be associated with protection from malaria in adults as individuals who developed clinical episodes had significantly lower levels of these antibodies, although the numbers were small and strong conclusions cannot be made. Much larger studies will be needed to rigorously test these associations. Interestingly, ADRB activity was not associated with protection, although the caveat about sample size of symptomatic malaria in this study should be clearly noted. This is in contrast to results obtained in

prospective cohorts of children in Kenya [531] and Senegal [140] in which ADRB activity was associated with protection from malaria. In a second study involving Kenyan children, ADRB activity in combination with growth inhibitory activity (GIA) was associated with protection from severe malaria [515]. The majority of adults (81%) had stable high levels of effector functions (above median) to at least one of the three assays resulting in a minimum breadth score of one. The breadth of immune response increased with age and individuals who experienced a clinical episode had a lower median score than those who were malaria-free, although this was not statistically significant. The trend towards a protective effect from malaria with increased breadth of effector functions has been observed in studies with Kenyan children where a combined GIA and ADRB activity was associated with protection from severe malaria [515]. No studies have looked at multiple effector functions in the same adults; however, it has been observed that the breadth and magnitude of antibody responses is associated with the protection from malaria in adults. These have primarily been in studies that evaluated the protection observed in adults in the context of; 1) the sterile protection achieved following experimental controlled human infection with sporozoites [271, 272, 533, 534], 2) semi-immune adults following natural exposure [268, 271, 535] and 3) the genetic resistance observed in the adults of the Fulani ethnic group [536]. It is likely that responses to multiple antigens resulted in an increased ability to elicit multiple effector functions, which is supported by the strong positive correlation ( $r=0.6$ ) between the breadth of antibody and immune effector responses (Figure 6.10B).

***Antibodies to novel antigens are strongly correlated with immune effector mechanisms***

Identifying the merozoite targets that elicit protective immune effector functions would aid in prioritizing antigens for vaccine pre-clinical evaluation and identifying the quality of vaccine-induced responses. In line with this, I evaluated the correlation between antigen specific levels and immune effector functions in the same individuals. A strong positive correlation (greater than 0.5) was observed between antibody levels to six novel antigens namely PF3D7\_0206200(*PfPAT*),



PF3D7\_073800.2, PF3D7\_1252300, PF3D7\_1308000, PF3D7\_1345100 and PF3D7\_1401600(PHISTb) and C1q-fixing antibodies suggesting a possible mechanism by which these antibodies function. Encouragingly, a similar strong positive correlation was observed between the well-studied antigens MSP1-19 and MSP-2 antibodies and the levels of C1q-fixing antibodies, which fits well with the following published observations [138]: 1) rabbit polyclonal sera raised against recombinant MSP1-19 and MSP2 (FC27 allele), 2) affinity purified antibodies against MSP-1 and -2 from Kenyan adults and 3) antibodies from MSP2-C1 vaccinee's [524] all demonstrated C1q-fixing ability [138]. In contrast, no C1q-fixing ability was observed using rabbit polyclonal sera against AMA1, MSP-3 and MSP-4 [138]. Similarly, a weak correlation was observed between antibody levels to these antigens and C1q-fixing ability.

ADRB levels were strongly correlated with antibody levels to MSP-2, PF3D7\_0206200 (*PfPAT*) and PF3D7\_1308000. Little is known about the contribution of antigen-specific antibodies to ADRB levels with the exception that depletion of MSP1-19 specific antibodies in serum from Senegalese individuals showed a slight reduction in ADRB activity [537]. Similarly, a weak positive correlation (Rho values of less than 0.5) between anti-MSP5 antibodies and ADRB activity was observed in a cohort of Senegalese individuals [538]. Lastly, no correlations were observed between antibodies to any of the 21 antigens evaluated and opsonic-phagocytosis levels. Although not directly comparable, these findings contrast with data in which anti-MSP2 and MSP3 affinity-purified immunoglobulins from Kenyan adults showed strong opsonic phagocytosis activity in a dose-dependent manner [139]. Interestingly, it has been suggested that opsonic-phagocytosis of merozoites is strain transcending and therefore targeted at conserved antigens or domains in merozoite antigens [530]. No differences were observed between opsonic-phagocytosis of wild-type parasites and those deficient of MSP3, MSP4, DBLMSP1 and MSP1-19 when measured in children, suggesting that these were not the dominant targets for opsonic-phagocytosis [530].

### ***Unanswered questions***

Although immune effector mechanisms were detectable in the majority of adults in all three surveys, the longevity and stability of these responses in the absence of exposure or in the context of decreasing transmission intensity has yet to be elucidated. More importantly, the immune effector functions elicited by antibody responses to novel antigens identified in this study require to be evaluated specifically. Although strong positive correlations suggest mechanisms by which these antibodies may function, these findings need confirmation. This would include evaluating antigen-specific responses obtained either from animal immunization studies or affinity-purified antibodies from humans. These antigen-specific antibodies can then be tested against the panel of assays that are available to evaluate antibody function. Furthermore, as the novel antigens have limited polymorphism in global isolates (Chapter 5), the degree to which these functional responses are effective against diverse parasite isolates require evaluation. Lastly, the contribution of the invasion/growth inhibition assay and the antibody-dependent cellular inhibition was not evaluated in this cohort due to the limited serum volumes available and the difficulty in setting up the ADCI assay. Testing these assays in the context of different studies where larger sample volumes are available is a clear priority.

### ***Summary***

I provide evidence that opsonic-phagocytosis and recruitment of C1q to merozoites is associated with protection from clinical episodes of malaria in adults, although conclusions are limited by sample size. Secondly, I show strong positive correlations between antigen specific antibody levels and recruitment of soluble C1q, particularly for three novel antigens whose antibody levels were associated with protection from clinical episodes in malaria. These targets include *PfPAT*, PF3D7\_1345100 and PF3D7\_1401600(PHISTb). These data provide supportive evidence that suggest the mechanisms by which antibodies to novel antigens mediate protection.

## CHAPTER 7

### Conclusions and recommendations

#### 7.0. Summary of findings

Antibodies are a major component of a host's natural defence against most pathogens including *Plasmodium* parasites. One major bottleneck to malaria vaccine discovery is identifying the specific targets of naturally acquired immunity in the >5400 proteins predicted to be encoded by the *P. falciparum* genome. The experiments and analyses presented in this thesis were aimed at addressing the above knowledge gap and I summarise the findings below.

First, I identified a set of merozoite proteins that were reactogenic with a pool of immunoglobulins from malaria immune adults. Second, I identified another set of proteins that may potentially be located on the surface of merozoites and therefore accessible to circulating antibodies. The data from these experiments were combined with bioinformatic predictions resulting in a list of potential targets of protective humoral responses. As predicted, the overwhelming majority of these targets had not been evaluated in the context of naturally acquired immunity to malaria. Of those that had been previously studied, several interesting features were noted:

- i. ***Known immunogenic antigens.*** Over a quarter of the proteins I identified had been evaluated in previous studies and shown to be targets of naturally acquired antibody responses. The identification of these proteins validated the overall approach, and added confidence that the unstudied proteins within the list were potential targets of protective immunity.
- ii. ***Roles in erythrocyte invasion.*** Previous transcriptional studies that evaluated the gene expression patterns during the *P. falciparum* intra-erythrocytic developmental stages following exposure to growth-inhibitory compounds, identified a set of >400 genes with a potential role in erythrocyte invasion [258]. Interestingly, 16.4% of the proteins I identified that had not been evaluated in the context of naturally acquired immunity were also identified

in this past work to be invasion-related. I subsequently demonstrated that antibodies to some of these targets were associated with protective immunity in adults, in line with their potential role in erythrocyte invasion.

- iii. ***Essentiality for asexual growth.*** A proportion of the targets I identified have *P. berghei* orthologues in which attempts to disrupt the genes were not successful [261]. These findings suggest that these targets may have essential roles in parasite growth or survival during the asexual stages, although essentiality cannot be confirmed without a conditional genetic approach. The findings from my analyses indicating that antibody responses to some of these essential targets were associated with protection from malaria warrant their further evaluation in pre-clinical studies.

Lastly, I used a cohort of longitudinally monitored adults living in a region that experiences perennial malaria transmission, to evaluate antibody responses to a set of potential vaccine candidates identified by my mass spectrometry and bioinformatics approaches. Antibody levels to six antigens appear to be associated with protective immunity, as adults who experienced a malaria episode had significantly lower levels of antibodies to these antigens. To examine potential protective mechanisms, I studied antibody effector functions that have previously been associated with protection from clinical episodes of malaria, namely interaction with neutrophils and recruitment of soluble complement proteins. These two mechanisms were strongly correlated with antibody levels to some of the novel vaccine candidates presented in this thesis, providing a potential mechanistic explanation for protection.

### **7.1. Recommendations for future studies**

This work could be extended in the future in two main ways: i) additional pre-clinical evaluation of the nineteen proteins presented in this thesis as potential vaccine candidates (19/22 proteins I obtained were found to be immunogenic and I evaluated only 11 antigens in the cohort studies) and ii) evaluating the remainder of the long list of identified proteins for their potential as targets of naturally acquired

antibody responses (I prioritized 27 of the 159 potential vaccine candidates identified using the down-selection criterion described in chapter 4).

#### ***7.1.1. Further pre-clinical evaluation of antigens studied in this thesis***

The antigens evaluated in this thesis as targets of protective immunity in adults should be evaluated further in the following ways:

- i. Examining antibody responses in other cohorts of adults exposed to *P. falciparum* transmission to confirm the findings from this work. These should include measuring the longevity of antibody responses to these antigens as well as effector functions using samples obtained either from adults living in highly seasonal malaria transmission settings or whom have travelled to non-endemic regions. In Kilifi, Kenya this analysis may be possible due to a unique “natural experiment” that has occurred over the past ten years. Two ethnically and culturally similar cohorts of children and adults with historically similar exposure to *P. falciparum* malaria have experienced a marked difference in their levels of exposure to malaria over the last 9 years [539, 540]. Analysing antibody responses in these individuals may provide insight into their dynamics particularly in the absence of exposure. In addition, experimental human malaria challenge facilities have been set up in Kilifi, Kenya, which will now facilitate the evaluation of both antibody responses and effector functions in adults, and correlate these with parasite growth rates and the development of clinical symptoms following experimental challenge with sporozoites. Similar studies should be conducted in individuals from varying geographical regions including West Africa and South-East Asia where *P. falciparum* transmission also occurs.
- ii. These targets should also be evaluated by measuring antibody responses in children from regions with varying transmission intensities to identify targets consistently associated with protection from severe, uncomplicated malaria and high parasite densities.

- iii. The antigen specific antibody effector functions elicited by the targets presented in these studies should also be evaluated further. The degree to which these responses are effective against a diverse set of parasite isolates will be crucial in selecting which of these targets should be prioritized for vaccine development.
- iv. Further analysis within individual antigens could be conducted to identify the epitopes that are important for protection. Short linear epitopes can be readily obtained as synthetic peptides and WHO has published guidelines for the production and quality control of synthetic peptides for use as vaccine constructs [541].
- v. Analyses to identify the role of polymorphisms within the newly characterised antigens and assess their potential effect on protective immunity should be conducted.
- vi. Animal models of *Plasmodium* infection also offer an approach to evaluate the immunogenicity and protective efficacy of potential vaccine candidates prior to clinical studies in humans[542]. This may include evaluation in either rodent malaria infections (*P. berghei* or *P. yoelii*) or using the recently developed humanised *P. falciparum* mouse models. In rodent malaria infection, vaccine efficacy can be evaluated by vaccinating mice with recombinant protein (the *P. berghei/P. yoelii* orthologue of the *P. falciparum* target), followed by *P. berghei/P. yoelii* parasite challenge and monitoring disease progression [543]. Alternatively, where applicable, mice may be vaccinated with the *P. falciparum* antigen and challenged with a chimeric rodent malaria parasite that expresses the *P. falciparum* protein [544, 545]. In a second approach, humanised mouse models continue to be developed that support *P. falciparum* blood stage growth and development [546-548]. While vaccination may not yet be possible in these immunosuppressed mice, the role of antibodies generated against a vaccine candidate can be evaluated using passive transfer studies of immunoglobulins followed by challenge with *P. falciparum* in these mice [549, 550]. Obtaining sufficient recombinant protein may occasionally represent an obstacle to the above

approaches. As an alternative, immunization of mice with expression libraries may represent a simple vaccination tool to quickly evaluate the protective efficacy of each of the identified targets [551]. Briefly, selected exons or open reading frames from parasite genomes e.g *P. berghei* orthologue of *P. falciparum* targets are cloned into eukaryotic expression vectors and directly used as DNA vaccines. Direct immunisation with DNA can generate both cellular and humoral responses and their protective efficacy can be evaluated by challenging immunised mice with the pathogen, in this case *P. berghei* [552].

- vii. Approaches have been developed to analyse the roles of genes across the *P. falciparum* life cycle. These include conditional approaches that can regulate the expression of a gene at the genomic, transcription or protein level providing information on the functions of these genes. These approaches could be applied to the antigens identified in this thesis to elucidate the roles of these antigens in erythrocyte invasion and/or parasite growth over the asexual stages of the life cycle. Several approaches are currently in use in *P. falciparum* to elucidate phenotype following regulation of expression of a gene that include: a) gene deletion by double crossover homologous recombination [553], b) gene editing using the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein-9 nuclease), c) gene editing using zinc-finger nucleases, d) conditional control of a gene using the dimerizable Cre recombinase (DiCre) system [554, 555] and d) use of the destabilisation domains (DD) to degrade target proteins after protein expression. These options should be evaluated and employed to elucidate the potential roles of the genes identified in this thesis.

### ***7.1.2. Evaluation of proteins identified but not analysed further.***

Due to the time constraints of my PhD, I selected 27 antigens for evaluation in the context of naturally acquired immunity. A total of 159 targets had been identified

using the selection criterion described in Chapter 4, leaving a set of 132 proteins that have not been evaluated for their role in protective immunity. Of these 132 targets, 92% are predicted to have an N-terminal signal peptide or at least one transmembrane domain indicating that they may potentially be secreted or located on the surface of merozoites and therefore accessible to antibodies. In addition, 12% of these targets are proposed to be invasion-related genes warranting their further evaluation as targets of protective antibodies. Lastly, 41% of these targets have *P. berghei* orthologues whose genetic disruption has been achieved and a phenotype following disruption deposited on the PlasmoGEM website [336]. Interestingly, 33 targets are classified as being essential for asexual growth while disruption of 9 targets resulted in a reduced growth rate over the blood stages. The *P. falciparum* orthologues of these targets represent attractive candidates for pre-clinical evaluation as targets of protective immunity. Below, I highlight experiments that may allow high-throughput screening of these targets:

- i. A *Plasmodium falciparum* expression library can be generated for these specific targets and used to screen for reactivity with carefully selected serum samples from individuals resistant to malaria infection and disease.
- ii. Alternatively, as highlighted above, expression library immunization may offer a rapid approach to testing the potential protective efficacy of these targets using animal models. These could be initially tested in pools consisting of 10-20 DNA vaccines and down-selected in a systematic manner.

Thereafter, targets with a positive phenotype can be evaluated further using the pre-clinical evaluation studies proposed in section 7.1.1.



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## APPENDICES

### **Appendix 2.1. Immunogenic *P. falciparum* antigens identified by 2DE using 3D7 lab isolate**

<https://www.dropbox.com/s/kfkme00hskte2m9/Appendix%202.1.xlsx?dl=0>

### **Appendix 2.2. Immunogenic *P. falciparum* antigens identified by 2DE using KIL9605 clinical isolate**

<https://www.dropbox.com/s/vsgpeui32lc69x9/Appendix%202.2.xlsx?dl=0>

### **Appendix 2.3. List of targets identified in 2D-immunoblots with phenotypic data available on PlasmoGEM**

<https://www.dropbox.com/s/gckhfsu84yzdfbp/Appendix%202.3.xlsx?dl=0>

### **Appendix 3.1. List of *P. falciparum* antigens identified in the 1st biological replicate of the surface shaving experiment.**

<https://www.dropbox.com/s/ro0cykoyh3bw9qo/Appendix%203.1.xlsx?dl=0>

### **Appendix 3.2. List of *P. falciparum* antigens identified in the 2nd biological replicate of the surface shaving experiment.**

<https://www.dropbox.com/s/zaz48063ofxkhjs/Appendix%203.2.xlsx?dl=0>

### **Appendix 3.3. List of *P. falciparum* antigens classified as putative surface proteins**

<https://www.dropbox.com/s/d70nbc1136gzbms/Appendix%203.3.xlsx?dl=0>

### Appendix 3.4. List of targets identified in surface shaving experiments with phenotype available on PlasmoGEM

<https://www.dropbox.com/s/g7ktqe1raor2ydu/Appendix%203.4.xlsx?dl=0>

### Appendix 3.5. List of *P. falciparum* putative merozoite surface proteins whose role in NAI, merozoite invasion or essentiality is unknown

<https://www.dropbox.com/s/ohdulwpj986jqc/Appendix%203.5.xlsx?dl=0>

#### Appendix 4.1. Subset of targets used to select novel antigens for immunoprofiling (N=222)

<https://www.dropbox.com/s/irawmfvegcbf2va/Appendix%204.1.xlsx?dl=0>

#### Appendix 4.2. The protein sequences for the 27 antigens selected for immunoprofiling.

## Glossary

Red= predicted N-terminal signal peptide and transmembrane domains or start methionine residue

Yellow- predicted N-glycosylation sites

Green= internal helices

1. **>PF3D7\_1105800**  
**MNWKKALS**LAGGAAAVVALTYMLMKDEDNHHDDNDDEKDKKKKDNKLSKDSNRIIKGDSMTREDLLQLLNEML  
 KLQSDMKNIVKDLIVVAKNNNYDFMAVYNVAKTYNTIDPLGKYQIEMPEFDKVVESYHFDPEVKETVSKLMSSQEN  
 YYS**NMS**ETTLTVSDKIIIEHHFMLNELYKIDPEFKKIPNKNEIDPKLIALVIQSIVSAKVVEEFF**NLT**SEDEVASIANQQYAL  
 TSNMELFARVNIQMOTIMNKFMGDHFKFMCDEKEGAY

>>PF3D7\_1105800 sent to GeneartAG  
DEDNHDNDNDENDKKKKKDNKLSKDSNRIIKGDSMTREDLLQLLNEMLKLQSDMKNIIVKDLIVVAKNNNYDFMA  
VYNVAKTYNTIDPLGKYQIEMPEFDKVVESYHFDPEVKETVSKLMSSQENYYSNMAETATLSVDKIIIEHHFMLNEL  
YKIDPEFKKIPKNKNELDPKLIALVQISIVSAKVEEENLASDVEASIANQYALTSNMEFARV  
NIQMOTIMNKFMGDHFHFKMCDKEGAY

[illegible]

NDQETQTD DDDINDSDIDENNNTNDKNNCHMYKKNCHMYKKNCKKYFLNSHYNNYLCKKQKKYKNKNDMGNK  
QKDNKKFKL NESMKNHFYNIFVKETVMLKKLYKVL FELF

>PF3D7\_0722200\_RALPI sent to GeneartAG

SSNDKNKINKNNMPLQRGASNLKDINHVEKKS NMESVNNNINNTA CSKNLNANKVPNV AHTGVNKKGETKNEE  
VEKEENENE EEEIEEENEENQQNEEDENEENQQNEEDENEENQQMNVDVSAEKKKELPEEKELQKNSEKNIN  
KEVIKDSNKDNTFNHFNKNINTSFNKNKNINNNNNNNNNNKA FIEKKEEAIDMKKTHCSNNNMCKYNNMNMNHS  
TNKNNNQIFEEESKQNVNKPINL KSLGNNTFKNDEKY NENDYKNNHDDDDKKKYTDDNEENIYEERNQKILLIHLK  
NIKELLNRQRQNFNNFLSFLSENYQSYEFYKSQKYQNGKNYIDKLDQOGELKNVA VVTHSFLDSKAANGKKDK  
NGVFVKLMNDQNDGDGDDTKDGDGDDTKDEDDHKNEDDHKNEDDHKNEDDHKNEDDHKNGDDNKNGDDHKNGDD  
NKNGDDDDNGKKS HSDISDIKNIIDTILQSDDITDEQKKYLEIHKILDLEEDVLNKEKEQLQLNKNIEVLMGKSDEL RNI  
AVNLKNGNGDNEASQRVDLAQNIVSNLLNFA VQLKNTGNIVYNNIQGGGELLQSIEKNIDKAENDLKKSTSVNTTFT  
PKNVNTEQN DQETQTD DDDINDSDIDENNNTNDKNNCHMYKKNCHMYKKNCKKYFLNSHYNNYLCKKQKKY  
KNKNDMGNKQKDNKKFKL NESMKNHFYNIFVKETVMLKKLYKVL FELF

3.

>PF3D7\_1014100

MNRIFYFCLFTILFWLSLVSG ENVNNKNCNEKNRKA ILLALLKNSLVDNKDY NNS EELKYALEHIQNSELYPKDSKKF  
DKFIDEFFSYNNIHV NFT DEEKRI LHISGVFKEFYVDVDNLNKDEMKEYFKKNYEKGLSLINLIVHSNLIIQQFDHDIID  
KKKVHEQNTNT NKT LEYISDNLNDLINFKNIHLLN NST GDFIILKLYTNYVNYINPYQTNPLNTPHYEHKNFHTKEH  
YIYDEEIVNPNMNDNINTHT EEDNVYVSATKGQKEETEKKENHENNAINPKYMN YETYYKKIFNAIFEQIDKL NKT  
LFEIKNK NNS ETNE NIS ESNSGNPELNNENSYSVKLSSSSPNSTNKESLIFPYTYNPNYMFRLTNNFKENDEGLKNEN  
NINNNEEDNQDNMNI VLGKIHNILKDFNINENIMTNKMSAPLIMTILNFFKKYMAENKFNLPMSSSEVENKI NKS NNK  
ALLQOSNKDTPHKKKKEIRNKKIOTKV DVIDEKT KKKIANTYVNVGQSGINGFFNFFDFREKSIDSNIFDLLHV MEDM  
KIFDIFQTIIFIQKFTENVCASYCM NIT DVLELSHYDMIFYDKMVHFHFKDGMMIKTDKKYLNLKEFENILNLLNINA  
NTIAL NCT CKFYVDV NYT YSEQYKMLHKGYLHKMNEFDYINNFSASYLLNQLIIFQDKFN YIKMNGKLPIDDPKNY  
NMNNVHD TAYYHNSRYFPTKDMPSLEDNFYEHLKYPDINTIHIY YNASPVKLNEVNDLKTIIIDEIKSKIFYINSYRVG  
DQFFPTYSNLGKDDHDL EHS AKNFY NIS NENGDN TFNNNNNNMNDNKKRMYNYNKHKDNDSRYTDNSNKNRDN  
KNRDYNNRNKDKNNTNRDNYNR YKDNYYNNSDNNNNYNERKRYIRKKTYNKL SYFNLPSLSKIYNNKIKGNSEE  
FSFDNELPEQTESFPLNKPQDHEAFYNLKKHHTNVYEPNDEEKQNEQKLKDQIKITS DILYKDIEENKNTEDVLLIETIT  
INN GTTSNTIENNKDSNKEAENSNT EQNDNNNNNDNNNNNNNNNNNDNKEEDMNENN NNS KVTGDSVENINEQT  
NNQYPNTEYNTIQRSINAKYL IFFFFKNLHVWKTDLFCQINYMNNYLSIQY NKT LTFDIN YDTNAV TIYFTD NIT Y  
VKVNL EYLVLLEKISLITFVEDLCSLFDTDKKNYK NLT EFLENTERINTFVRNHMMLSNEQFINKNYAKELAEIST  
SNLFYPKKDILRSTPYNNIILDEKDIYQTIIFYMDDMLTEKMV NDT WITPYAFVVSYSKKDMQGNNIKIEQNK NIT  
YSRSAIDKYVHYEYKRISENLNRRFMESNSNAPQFNENYKEYTIIYNDNPSPMPNIVLTTTINVFVNSFLQSIMEMLLNI  
KANQQFFSYKGFIPINAFITLENKINYIFFNYIPL ENYVNNGDALDFRNP

>PF3D7\_1014100 sent to GeneartAG

ENVNNKNCNEKNRKA ILLALLKNSLVDNKDY NNA EELKYALEHIQNSELYPKDSKKF DKFIDEFFSYNNIHV NFA DE  
EKRI LHISGVFKEFYVDVDNLNKDEMKEYFKKNYEKGLSLINLIVHSNLIIQQFDHDIIDKKKVHEQNTNT NKA LEYIS  
DNLNDLINFKNIHLLN NSA GDFIILKLYTNYVNYINPYQTNPLNTPHYEHKNFHTHEHYIYDEEIVNPNMNDNINTHT E  
DNVYVSATKGQKEETEKKENHENNAINPKYMN YETYYKKIFNAIFEQIDKL NKA LFEIKNK NNA ETNE NIA ESNS  
GNPELNNENSYSVKLSSSSPNSTNKESLIFPYTYNPNYMFRLTNNFKENDEGLKNENNINNNEEDNQDNMNI VLGKI  
HNILKDFNINENIMTNKMSAPLIMTILNFFKKYMAENKFNLPMSSSEVENKI NKA NNKALLQOSNKDTPHKKKKEIRN  
KKIOTKV DVIDEKT KKKIANTYVNVGQSGINGFFNFFDFREKSIDSNIFDLLHV MEDMKIFDIFQTIIFIQKFTENVCAS  
YCM NIA DVLELSHYDMIFYDKMVHFHFKDGMMIKTDKKYLNLKEFENILNLLNINANTIAL NCA CKFYVDV NYA Y  
SEQYKMLHKGYLHKMNEFDYINNFSASYLLNQLIIFQDKFN YIKMNGKLPIDDPKNYNMNNVHD TAYYHNSRYFPT  
KDMPSLEDNFYEHLKYPDINTIHIY YNASPVKLNEVNDLKTIIIDEIKSKIFYINSYRVGDQFFPTYSNLGKDDHDL EHS  
AKNFY NIA NENGDN TFNNNNNNMNDNKKRMYNYNKHKDNDSRYTDNSNKNRDNKNRDNYNNRNKDKNNTNRD  
NYNRYKDNYYNNSDNNNNYNERKRYIRKKTYNKL SYFNLPSLSKIYNNKIKGNSEEFSDNELPEQTESFPLNKPQ  
DHEAFYNLKKHHTNVYEPNDEEKQNEQKLKDQIKITS DILYKDIEENKNTEDVLLIETITINNGTTSNTIENNKDSNKE  
AENSNT EQNDNNNNNDNNNNNNNNNNNDNKEEDMNENN NNA KVTGDSVENINEQTNNNQYPNTEYNTIQRSINAK  
YL IFFFFKNLHVWKTDLFCQINYMNNYLSIQY NKA LTFDIN YDTNAV TIYFTD NIA YTVKVNL EYLVLLEKISLITF  
VEDLCSLFDTDKKNYK NLA EFLENTERINTFVRNHMMLSNEQFINKNYAKELAEISTSNLFYPKKDILRSTPYNNI  
ILDEKDIYQTIIFYMDDMLTEKMV NDA WITPYAFVVSYSKKDMQGNNIKIEQNK NIA KYRSAIDKYVHYEYKRISE  
NLNRRFMESNSNAPQFNENYKEYTIIYNDNPSPMPNIVLTTTINVFVNSFLQSIMEMLLNIKANQQFFSYKGFIPINAFI  
TLENKINYIFFNYIPL ENYVNNGDALDFRNP

4.

>PF3D7\_1455300

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SLCNI AKDDIVFNKSRKRDKQSRSDSKKGNE MNTDYMYSNNNNNNIYANYVFKQPNEIS NGS ILYTLRNNNNNNNNY  
NNNNVVTPLYIN NQT KTNDNLINILSPNYVDPAPPIVLKQNVLPQLYIRQPPTVIVTNEPRPLVINPPANII FKNKSP  
QPIYV NST RPNIIKNDPPV VQNPINMDTPVQLDMPTE NIT INKETINYPYVMNIKKDSFLDNRRNYYLKGVSSTNASV  
SPTNV IYVESNNNNNNMNDINQQNIPNFYMMNNNQTPHLHQTTNAYATIPPINNHIQIQQTNTVQYIQPNYEQVITQ  
VDQNRNLFNQQLVGSTVMQNMTPQGVNTLNYPATINTVCVPQTISNTNTTSQQVQYIPQTQAYAPYNNVQ NITIY  
ERNYVPQIAQNTMTNNV VQQYVPSTTTMVQENVQNT PSSQYRILVPNNDNIQSPNIIRHYN NIS NTKNNVNKAIVQPT  
Y NNS EILPSCSPSGCASRNKVTHVQNVRRNSYINPHSHSMHECPKKVQIARPMHDQNLRTYSLCR

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VSPTNVIYVESNNNNNNMNDINQQNIPNFYMMNNNQTPHLHQTTNAYATIPPINNHQIQTOPTNTVQYIQPNYEQVIT  
QVDQNRNLFNQQLVGSTVMQNMTPQGVNTLNPATINTVCVPQTISNTNTTSQQVQYIPQTQAYAPYNNVQNTAI  
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5.

>PF3D7\_1460600\_ISP3

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VIKFNDCILHRNSCETLLESEQNLLKSPKVIIGIRLIST LKAIAFSMDSPGEARIFNDFLQKYCLNA

6.

>PF3D7\_1468100

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KNKYNNLEYFENKEKNLMLPNNNNNNINMNSNMNGHINSHLSNKKGNKNNNQHCNINHNINNNNNNNNNNFV  
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HDIKKLEKDPNRNVVPDASGLARIEAAFGLSKSSYDFINNSQNSTVG NLSNVLFNNRNLNSALLNNQFYSNLSNV  
TICLFGGEYCEISKTHKERTLLDDMWIFKVHNNVHISANSNDGTSYNNGMMDKTQNDKNHTNNNNNNNNNNSSSEK  
NNNNNNNNNNNNMNTFNNNNMNTFNN  
DEEEELMKVIKKNIESYKMNNIKNILISSVFGRIKWLMKNESPHAYLYGHSIVKFGNKLVMFGGSGNGKNKKIPFHTTL  
TFSLIYYNYKLLPLSGNCPPEEREHGTTHLVSLHNGLSVFLFGGSGNENIYNDIYLLDMETRWTRRSVKGKIPLPRDQ  
HSSLVYPKACEHVRGEKPNLTEGVIIFFGGKCLYNNISIVSLNDMWIFSFSILWIRINYLCDDIPMGRFGMNLVWSDTN  
TICLFGGEYCEISKTHKERTLLDDMWIFKVHNNVHISANSNDGTSYNNGMMDKTQNDKNHTNNNNNNNNNNNNSSSEK  
HNMHSNDKEYADNVKDKYNNNNKFTMVGEWYRENYEGDIGCRSNYSVFITQRHQDFKGAEPKTIERLMILCSGIT  
YVYKDNKQKIVSTDEVFVYFFSQKKWYLLKGKLCNDEYLYNGRQRHVGCFFESKNVLGRANRNPVPCVFIQGGFK  
KNSVFGDAWLLSLTGENPLRIQEYDTSRERISTTQMPLYYFRDTHSISLLYSFCTLQKWLFGAFANLVDNCVHAHNPA  
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LELMNHCEAKELATPLCMWKLPNKELINRNIAKSEHRHHQKLLMSYTPFNPSLLAEQINILGTYSGTRLLYWDNR  
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>PF3D7\_1468100 sent to GeneartAG

SKINGANIDEMNHIEQNLSANNQLNDDNYNAAKNFSLSNYNLDLIKCLQDDSGIYFILDPSKAINEKKQSDVYDIFV  
PEDTEGVTLHVQKMNDYKICIGVSITKKGIDINSCTRIFNSKISDWLEQLFVFNKNLINNGLQMNEKMKKIPNNAAVL  
NTNNVNSGASFMNGDINGNINIPAINYKNDKFINKGSNINIINEGSNLAHSKNTNYNNMKRASGTSNLQDTTTNLI  
KNKYNNLEYFENKEKNLMLPNNNNNNINMNSNMNGHINSHLSNKKGNKNNNQHCNINHNINNNNNNNNNNFV  
GNHNLPSQMOKQNRNLNLVNNNNNNKNAHSNKA NTMNFKNMNFDEKNSKLFNNIAASSLLMNKNILSNVNALAA  
QISLGASSEFMKNNKLGVGHTNNNSQKNNTYNLHNHLQNELFNLPHLQNNLMFNNNNNKAKLHQLQNSQNNNV  
VHQHIQONQANANQINQSYNDNFA YRPTLFNLLEVLANHNIA TPDQRCVIRGIVTDFLNNELPHGKIYAYIGAVVGH  
DILHDIKKLEKDPNRNVVPDASGLARIEAAFGLSKSSYDFINNSQNSTVG NLANVLFNNRNLNSALLNNQFYSNLS  
NVANNALNANNLLNNNERVSLDNDKILSSKAAADISNLLKYAKNEKMSMSANNVMNFRNKKGANFNLLNLNRN  
DPNNNNNNNNNNNNNNMNTFNNNNMNTFNN  
NSFSDEEEELMKVIKKNIESYKMNNIKNILISSVFGRIKWLMKNESPHAYLYGHSIVKFGNKLVMFGGSGNGKNKKIPF  
THTLTFSLIYYNYKLLPLSGNCPPEEREHGTTHLVSLHNGLSVFLFGGSGNENIYNDIYLLDMETRWTRRSVKGKIPL  
RDQHSSLVYPKACEHVRGEKPNLAEGVIIFFGGKCLYNNISIVSLNDMWIFSFSILWIRINYLCDDIPMGRFGMNLVWS  
DTNTICLFGGEYCEISKTHKERTLLDDMWIFKVHNNVHISANSNDGTSYNNGMMDKTQNDKNHTNNNNNNNNNNSSSE



KNHNMHSNDKEYADNVKDKYNNKKFTMVGEWYRENYEGDIGCRS **NY**ASVFITQRHQDFKGAEPKTIERLMILCS  
GITYVYKDNKQKIVSTDEVFVYFFSQKKWYLLKGKLCNDEYLYNGRQRHVGCFFESKNVLGRANRNPVPCVFIQGG  
FKKNSVFGDAWLLSLTGENPLRIQEYDTSRERISTTQMPLYYFRDTHSISLLYSFCTLQKWLFAGAFANLVDNCVHAHN  
PAENVFIKYLTPHEDGMLSIQDDGEGLDNFAMNRVLMRYGNYKYQDNSSVVLVYNSGTNIKHALPNDNFINKG  
DYNDYQYENEYSNKRNTSSKNVLLDDASSALLPHKKKQK **NTA**DNDKNNNNNNNENDSNNEEKD **NIA**GDHNKNGE  
DENKVGSHNAYDNDQNGYVSDYYTKNYDQELFYENNVNIFDVKYGVGFKMSFARISSSCAIMSRTFTNIGIGLL  
SLELMNHCEAKELATPLCMWKLPNKELINRNIANKSEHRHHQKLLMSYTPFNPSLLAEQINILGTYSGTRLLYWDFR  
DDMDFIIFSPLNNNIYLSLSSPLSVDEIKYNNKKKNNNNKNNKMLNDDNNNKQVSCVEDVKLNNRADIKRKGNFDEE  
NVKKVKVQHDESCANVYAKNDEENEDSQLVTNEGSDKDASTKDENMKKEHPNEKDDNNNNNNNNNNENGDDNN  
KSYNDQIFKENEYQYY **NSA**IKKLNLFYNSHLNTSISKDYKASQIFPLWDHPKDSIDYCLSTYLYWLYLRRNTNIF  
LQNTLLIPTCMRKNDNDVNSEKKKKKKKKKKKKLNAKKKNLQEE **NTA**EQTSLSKDSNSNKDSEENVNLNQYN  
DEERNSKRRNNDDENESSSTSNRDKSDADNMNDVIKNDNKIGKEEMHKIKFEEALEYGISQESSRKNMSMKYKEE  
DDEEEEDDDDEEDDDDE  
NQDENRDENQDESQENQENQENQDDNENEEKPNEGSNENEEKPNDSIENNEADKEAKSDENNNSSNNNGEENI  
ENTNECKQKQKQEDNEVQEVHEIRTRTRVKNKELNSKKKKNVDSSTDSKNNDESEKTRQGSYVNTYMDGLKIKDE  
YYINNTSKYTLNFKLKLHKMVEFHYLFTPSDYEYGFMSMGLNDNNSPSIEVNRVCETGILLYYKNLRLDAP  
FIDTAYNLALAYPPNPSLYEGNLYKYALTIVVNPVYWLKPSISKQEFIHENNYAFLVFKKKLIGLIKHYLFICQDNVK  
LRKWRESRDLKLKRYLDKSSFSFDKSKNESDNDENDYKITNQEKENAEQSEKEGKGKNAQSPNNDNDQNEVVGAN  
EREDDSTNRNYNNGKSSNEEENEGTPADNEDENDSSNNSNSNQEQKEKILENDEEEATDDNEEKNEEEKNDDN  
EEEEYENNDNEKYNNKNSDDVNEDNKSLEDAEEEDTI

7.

>PF3D7\_0525800\_IMC1g

**M**CSTNKNLACCKGDNVFDGQING **NES**YPQVVNKQLPPKVLEPIIQNKIVEIPKEVYLEKIVEVPQIKTVERIVEQIRPVI  
KYKNVYKPKIVYVEKVKNVDKIYQEKIVEVPQIKTVEKIVEVPVYVNRERITVPRYMVVEKVIPVLKTSKRESIMEV  
PEVNCPHIDISKEVEDKEEIPINELKE **NOT**ISLADEKEIQILNDLTSQKVDS **NAT**INMEGEQDTTVDITITQENFCGTVSCN  
FLPNYP **NFS**KIGNPLCKGGPEKEKRFSSISYKSKDSGFPSIRIAKTPQMFQRNLVCSYA

>PF3D7\_0525800\_IMC1g sent to GeneartAG

CSTNKNLACCKGDNVFDGQING **NEA**YPQVVNKQLPPKVLEPIIQNKIVEIPKEVYLEKIVEVPQIKTVERIVEQIRPVIK  
YKNVYKPKIVYVEKVKNVDKIYQEKIVEVPQIKTVEKIVEVPVYVNRERITVPRYMVVEKVIPVLKTSKRESIMEV  
EVNCPHIDISKEVEDKEEIPINELKE **NQA**ISLADEKEIQILNDLTSQKVDS **NAA**INMEGEQDTTVDITITQENFCGTVSCN  
FLPNYP **NFA**KIGNPLCKGGPEKEKRFSSISYKSKDSGFPSIRIAKTPQMFQRNLVCSYA

8.

>PF3D7\_1343700 KELCH PROTEIN

**M**EGEKVTKANSIS **NFS**MTYDRESGGNSNSDDKSGSSSE **NDS**NSFM **NLT**SDKNEKTENNSFLL **NNSS**YGNVKDSLLE  
SIDMSVLDSNFDSSKKDFLPS **NLS**RTFN **NMS**KDNIGNKYLNKLLNKKKDDTTITNENNNINHNNNNN **NLT**AN **NIT**NNLIN  
NNMNSPSIMNTNKKENFLDAANLINDDSGLNLLKKFSTVNNV **NDT**YEKKIETELSDASDFENMVGDRLRITFINWLK  
KTQMFIREKDKLFDKKELEMERVRLYKELENRKNIIEQKLHDERKKLDIDISNGYKQIKKEKEEHRKRKFDEERLR  
FLQEIDKIKLVLYLEKEKYYQEYKNFENDKKKIVDANIATETMIDINVGAIFETSRLTLTQQKDSFIEKLLSGRHHVT  
RDKQGRIFLDRDSELFRIILNFLRNPLTIPIPKDLSESEALLKEAEFYGIKFLPFPLVFCIGGFDGVEYLSMELLDISQCC  
WRMCTPMSTKKA YFGSAVLNLFYVFGGNNYDYKALFETEYDRLRDVWYVSSNLNIPRRNNCGVTSNGRIYICIG  
GYDGSSIIPNVEAYDHRMKAWVEVAPLNTPRSSAMCVAFDNKIYVIGGTNGERLNS  
IEVYEEKMNKWEQFPYALLEARSSGAAFNYLNQIYVVGIDNEHNILDSVEQYQPFNKRW  
QFLNGVPEKKMNFGAATLSDSYIITGGENGVEVLNSCHFFSPDTNEWQLGPSLLVPRFGHS VLIANI

>PF3D7\_1343700 KELCH PROTEIN sent to GeneartAG

EGEKVTKANSIS **NFA**MTYDRESGGNSNSDDKSGSSSE **NDA**NSFM **NLA**SDKNEKTENNSFLL **NNAA**YGNVKDSLLE  
SIDMSVLDSNFDSSKKDFLPS **NLA**RTFN **NMA**KDNIGNKYLNKLLNKKKDDTTITNENNNINHNNNNN **NLA**ANN **NIA**NNLIN  
NNMNSPSIMNTNKKENFLDAANLINDDSGLNLLKKFSTVNNV **NDA**YEKKIETELSDASDFENMVGDRLRITFINWLK  
KTQMFIREKDKLFDKKELEMERVRLYKELENRKNIIEQKLHDERKKLDIDISNGYKQIKKEKEEHRKRKFDEERLR  
FLQEIDKIKLVLYLEKEKYYQEYKNFENDKKKIVDANIATETMIDINVGAIFETSRLTLTQQKDSFIEKLLSGRHHVT  
RDKQGRIFLDRDSELFRIILNFLRNPLTIPIPKDLSESEALLKEAEFYGIKFLPFPLVFCIGGFDGVEYLSMELLDISQCC  
WRMCTPMSTKKA YFGSAVLNLFYVFGGNNYDYKALFETEYDRLRDVWYVSSNLNIPRRNNCGVTSNGRIYICIG  
GYDGSSIIPNVEAYDHRMKAWVEVAPLNTPRSSAMCVAFDNKIYVIGGTNGERLNSIEVYEEKMNKWEQFPYALLE  
ARSSGAAFNYLNQIYVVGIDNEHNILDSVEQYQPFNKRW  
QFLNGVPEKKMNFGAATLSDSYIITGGENGVEVLNSCHFFSPDTNEWQLGPSLLVPRFGHS VLIANI

9.

>PF3D7\_0822900

**M**EVLQNMNRMDRIIEIQNLKNIINNKLKNVNDVDGYMRQLVDLYLKSQKSCKNLGSKKGKSNSKIRSVSRKS  
ERKINVKGIEKMQHYYENNDQNNNNKNNNDNNNNYDNNNNNNNDNNNNNNNDNNNNNNNDN  
NNNNYD **NNS**KQSFSQK **NSS**ENYD **NGS**GSDDEEKVNNYNEENE **NNT**QKKKKKKKKGNKKNNKNNKNSDKS  
ETNDEEEVTVEMEEKENIIEQLNEQQNEKLDEQLNEQPNEFDEQPNEFDEQPNEFDEQPNEFDEQPNEFDEQP  
NEFDEQPNEFDEQPNEFDEQPNEFDEQPNEFDEQPNEFDEQPNEFDEQPNEFDEQPNEFDEQPNEFDEQP  
EQSNNTNKDEPNTSYVNTENVIDQGKG **NNS**ECGNIVEEQNGDSLDERKEKNMKS AIRFMKESNYEDPHLNACLS  
DSNIDGVNINEELKVKDILSNPFTTSSYNEENEKVSNMEMHSNNIE **NIS**NCIQKKKTQINLNNVLKELKFKSANNI  
KYGRDNNFDDITEKYINDGSDDLETYKKNSSINDEEYPHYTNFVKHYVTKVGIYNNMEKNKLNFNVPQSQYGINHLY  
NMNKNSTYDTSNFQYKNSYSTTNEEQEKDKREYGFNNCM **NYT**NI **NCS**NINQSYSNMIRNYNTMVNDHNI **NCT**KTNI



NCTNPKLDCLSKSFSKIRKINLNCNSAYYPHCTYSNPLKSIKKYSCTNTKNYINEYNCTKNATSMPLPDFSCMNGVEIMK  
KHINIDNIKKHVNLENVKKNMDDLKNVKKLINLENIKNKINFENVKERVNIKNVTDHINIESISKVVECFDNNYYDIFEE  
AYKNYINKYVNNVKNITKEKKEYYKKNVNCNYELINKNLLKVAANNINNLLNNMYLKNKSKLQGLNTKKYIAFNHN  
KYVRFNLNSCYEKKMNSVKKKIELNKKFLNNYFHACYFECSDNPMLSNDISRVSCEPYQNSNEQYHQKQSSMTNSIN  
SKSNIYNNSSSHKLLNTEKKKNTSNDSEQINNLSFPNSNKEFNSNSEIDISCFNNGVKTfKYMKGIFEDSGGAENEIHY  
DQEKMYFDLNDNAYINVKGNLYFNTKDKLFYDIEYIKDISYDNVKNLNFHFGNYLITEKYAPCKII  
MKMCAHINQFMYNLLGVAKYDIYYKHKDNLVEIIHK

>PF3D7\_0822900 sent to GeneartAG

EVLQNMNRMDRIIEIQNLKNIINNKLKNVNDVDGYMRQLVDLYLKSQKSKSKNLGSKKGSNSKIRSVSRKSE  
RKINVKGIEKMQHYENNDDQNNINNKNNDNNNNYDNNNNNNNDNNNNNNNDNNNNNNNDNNNNNNNDNN  
NNNNYDNNNAKQSFQKNSAENYDNGAGSDDEEKKNNYNEENEENNAQKKKKKKKKGNKKKNKNKNKNSDKSE  
TNDEEEVTVEMEEKENIEQLNEQQNEKLDEQLNEQPNENFDEQPNENFDEQPNENFDEQPNENFDEQPNENFDEQ  
NENFDEQPNENFDEQPNENFDEQPNENFDEQPNENFDEQPNENFDEQPNENFDEQPNENFDEQPNENFDEQ  
EQSNNTNKDEPNTSNYVNTENVIDQGGKNNACGNIVEEQNNGDSLDERKEKNMKSARFMKESNYEDPHLNACLS  
DSNIDGVNINQNEELKVVDILSNPFTTSSYNEENEKVSNNMEMHSNNIENIANCIQKKKTQINLNNVLKELKFKSANNI  
KYGRDNNFDDITEKYINDGSDDELETYKKNSSINDEEYHYTNFVKHYVTKVGIYNNMEKNKLNFNVPSSQSYGINHLY  
NMNKNYSYDTNSFYQNKYSSTTNEEQEKDKREYGFNNCMNYANINCANINQSYSNMIRNYNTMVNDHNIINCAKNTI  
NCAKNTIINCAKNTIINCAKNTIINCAKNTIINCAKNTIINCAKNTIINCAKNTIINCAKNTIINCAKNTIINCAKNTI  
KHINIDNIKKHVNLENVKKNMDDLKNVKKLINLENIKNKINFENVKERVNIKNVADHINIESISKVVECFDNNYYDIFEE  
AYKNYINKYVNNVKNITKEKKEYYKKNVNCNYELINKNLLKVAANNINNLLNNMYLKNKSKLQGLNTKKYIAFNHN  
KYVRFNLNSCYEKKMNSVKKKIELNKKFLNNYFHACYFECSDNPMLSNDISRVSCEPYQNSNEQYHQKQSSMTNSIN  
SKSNIYNNNSASHKLLNTEKKKNTSNDSEQINNNSLAFPNNSNKEFNSNSEIDISCFNNGVKTfKYMKGIFEDSGGAENEI  
YDQEKMYFDLNDNAYINVKGNLYFNTKDKLFYDIEYIKDISYDNVKNLNFHFGNYLITEKYAPCKII  
MKMCAHINQFMYNLLGVAKYDIYYKHKDNLVEIIHK

10.

>PF3D7\_1401600 PHISTb

MVSYNEYKLCLNPRLDMKRKNYKYNVHEGIIRKGAKWCSMNKAHRSRFFICICVLLHMMVFGVCYKVEGSVHV  
TEPNSSSSRSLYENKGVYESNVEVHDNFLNKLKLYMSPNVENNGIKEDNVFGNTDLGGMESHEKFEKIRSSSTLSQG  
NYSNFKDGGYEYSELADSMTEDEIDKEVDKLMETNNNKEKVYSLWWQIMRNERVKYVMIMKNLYNFFLKLKKK  
YVNDNSFSEKQWNICKKRVKKNRLNYEKSINTTFLNWCNSLLLRKDYRKLKANRLKWELSIKTEKACRHNMLQ  
AFEEKVKAQSTKLDDLVDYVTEHIRKNKKKKKITKDGDCYSTGPNGQYNECMKTSYIDAQSDALVEDEVTEEE  
SDESVKKEEEEEEEEEKGVDDVAEDEDALKDSTASGDSSTS  
RYTVLSADSAEYMESDILSVLDVDPVNGPASARSVYVQAYTSEPKEMLYRPKIRYVTI

>PF3D7\_1401600 PHISTb sent to GeneartAG

KVEGSVHVTEPNSSRSLYENKGVYESNVEVHDNFLNKLKLYMSPNVENNGIKEDNVFGNTDLGGMESHEKFEKIR  
RRSSTLSQGNYSNFKDGGYEYSELADSMTEDEIDKEVDKLMETNNNKEKVYSLWWQIMRNERVKYVMIMKNLY  
NFFLKLKKKYNVNDNSFSEKQWNICKKRVKKNRLNYEKSINTTFLNWCNSLLLRKDYRKLKANRLKWELSIK  
TEKACRHNMLQAFEEKVKAQSTKLDDLVDYVTEHIRKNKKKKKITKDGDCYSTGPNGQYNECMKTSYIDAQSDAL  
VEDEVTEEE  
NIADESVKKEEEEEEEEEKGVDDVAEDEDALKDSTASGDSSTSRYTVLSADSAEYMESDILSVLDVDPV  
GPASARSVYVQAYTSEPKEMLYRPKIRYVTI

11.

>PF3D7\_1407800\_PM4

MALTVKEEEFSNTLIKNASAFDRCLKLGNLKNLKIQKKLQFLYLILFVLITGVFFFLIGNFYSHRKLQYQVIKNTKHTTIG  
FKIDRPHDKVLSSVLKNKLSTYVKESFFKSGYAQKGYLGSENDSELDDVANLMFYGEGQIGTNKQPFMFIFDTGS  
ANLWVPSVNCDSIGCSTKHLVDASASKSYEKDGTKEISYSGTGRGYFSKDVISLGDLSLPYKFIEVTDADDLEPIYS  
GSEFDGILGLGWKDLSIGSIDPVVELKKQNKIDNALFTFYLPVHDKHVGYLTIGGIESDFYEGPLTYEKLNDHLYWQ  
IDLDIHFGKYVMQKANAVVDSGTSTITAPTSFLNKFFRDMNVKVPFLPLYVTTCDNDDLPTLEFHSRNNKYTLEPEF  
YMDPLSDIDPALCMLYILPVDIDDNTF ILGDPFMRKYFTVFDYEKESVGFAVAKNL

>PF3D7\_1407800\_PM4 sent to GeneartAG

SHRKLQYQVIKNTKHTTIGFKIDRPHDKVLSSVLKNKLSTYVKESFFKSGYAQKGYLGSENDSELDDVANLMFYG  
EGQIGTNKQPFMFIFDTGSANLWVPSVNCDSIGCSTKHLVDASASKSYEKDGTKEISYSGTGRGYFSKDVISLGD  
LSLPYKFIEVTDADDLEPIYSGSEFDGILGLGWKDLSIGSIDPVVELKKQNKIDNALFTFYLPVHDKHVGYLTIGGIES  
DFYEGPLTYEKLNDHLYWQIDLDIHFGKYVMQKANAVVDSGTSTITAPTSFLNKFFRDMNVKVPFLPLYVTTCDNDDL  
PTLEFHSRNNKYTLEPEFYMDPLSDIDPALCMLYILPVDIDDNTF ILGDPFMRKYFTVFDYEKESVGFAVAKNL

12.

>PF3D7\_0730800.2

MVHFTKILAVTCFAATFQCSNNVILSKSYDMNKKNLNNVGFRRNRILSSKENREEPGTSGETTSGANGNKANIPDLN  
FINQTTSSCGNLGNLLAPLLKGIKSNLFSKYVNEDGTTKPNAPTAQSLTSIVKGMHDIKPQMEALKEGIKKNIEEEGE  
ESIKKDCDALKDGFKNIMKSFGGDFSNVQGATGIPGKLFKGLNEDESKTTENADIKDEIDILLEDNTDDDDDDDDG  
DDDDDDNNDDDDDEKEKEKEKEKEKEKGGKGGKGGKKKENKETSDDNNKNAGTSTLRGKKKKKF

>PF3D7\_0730800.2 sent to GeneartAG

SNNVILSKSYDMNKKNLNNVGFRRNRLSSKENREEPGTSGTTS GANGNKANIPDLDNFINQTTSSCGNLGNLLAPLL  
KGIKSNLFSKYVNEDGTTKPNAPTAQSLTSIVKGVMDIKPQMEALKEGIKKNIEEEGEEESIKKDCDALKDGFKNIMK  
SFGGDFSNTVQGATGIPGKLFKGFLEDESKTTEENADIKDEIDILLEDDNTDDDDDDGDDDDDDNDDDDDEKEKEK  
EKENEGKGKGGKGGKKKENKETSDDNNKNAGTSLTRGKKKKKF

13.

>PF3D7\_0831400

MMRLYTNVFLFIIVLCILSPGNNPLI KYVEIKEGIN NLS IHAHLRLLTEIME NCS LNFRLNDKNGEHDEEEERKCD  
DDQHSENQEHECDNDQHSENQEHECDNEQNDKCEES NNTDQADVNYLSMPIQIPYLRNEIMDDNYYSRYFDSNI  
MRTDEVTTTRLNDQNLIKCLCKLKAGKYINEAKIKYAHEIDSMKLTDKVKDIFKEFMRYIWKRYPSHQLRLYKKI  
KKDIEKYTRNHVICSLIDFETIEDELYYLIYPNREMYMSFDTAVNTKRRKKIKKLMREKARKDKEKNG YI

>PF3D7\_0831400 sent to GeneartAG

KYVEIKEGIN NLA IHAHLRLLTEIMENCAL NLFRLNDKNGEHDEEEERKCD DDQHSENQEHECDNDQHSENQEHEC  
DNEQNDKCEES NNA DQADVNYLSMPIQIPYLRNEIMDDNYYSRYFDSNIMRTDEVTTTRLNDQNLIKCLCKLKAG  
KYINEAKIKYAHEIDSMKLTDKVKDIFKEFMRYIWKRYPSHQLRLYKKIKKDIEKYTRNHVICSLIDFETIEDELYYLI  
YPNREMYMSFDTAVNTKRRKKIKKLMREKARKDKEKNG YI

14.

>PF3D7\_0925900

MIKKAVALFFIFFLFTIIRA SPGNDNNVNNNDGVVHLSKNIDVKNLQGTIFYEAT NAS DKIFPGLACRCKTYEFSGLK  
RDGNLGYVLI NFS CARNFIFGEKKSEMTEFKLILNKPLDE NTT TVEEFNASIYLVQGNQQILLNGNINIY AELNEQNEFE  
HLILGGQKSIEMIMSKYRT VLLDTYNKLNLSYLAGYEPSLLTWPFIQTDQTFCD

>PF3D7\_0925900 sent to GeneartAG

SPGNDNNVNNNDGVVHLSKNIDVKNLQGTIFYEAT NAA DKIFPGLACRCKTYEFSGLKRDGNLGYVLI NFA CARNF  
IFGEKKSEMTEFKLILNKPLDE NTA TVEEFNASIYLVQGNQQILLNGNINIY AELNEQNEFEHLILGGQKSIEMIMSKY  
RTVLLDTYNKLNLSYLAGYEPSLLTWPFIQTDQTFCD

15.

>PF3D7\_1025300

MLLYFVFIWSFLLCDI YETLNIRKSIDRKLTHLNNNIECAL NIS NYRNKKSKNLQFIGNNKIVKKCKNKYSNKVHLFG  
FSSKEEINNKGKKSLSNIEKEYKNGNIFLYTKLFVNNEEKKFIIDLCSDHSFIFEKRLNEELLKCNNNNNNNNIFTQND  
EQKSPKNEGLNLQYEKKDIENIKELFHL NNT QVDIKKVVNIKHEKEKQFPFYVINNEELDMNFGIIGFNFFKNYDFT  
VLDTIQMKILLNWNEDGINTEDPNYDNIEYEKQKNGEQDQKEHEYYKINLYDYFNNIKYFNIIYVDNHLKYGIVDTG  
TLNTILL NNS NIYNTTTDYNKDKTSTSIIESVLKNKYKV NKT NINHINIITKDNHKIPVELNKYIYSGNLEYINKDVILL  
GLDFLLNKKLIFDLKNNILIPKKYL NNT NEFNKKYDALNIGITNVQENQKSQNDNGNNHMDQIKIEDKCKEINYQLK  
AKELNFVRITKELENDNVYMKDCKSTNDVIHKYAIKLLYGSNYLKKNDKNIEYEKRYNEVTEFFKKLNNEEKQNM  
NKIVNSVLLLR NDT IHDNNNNNNNNNNIINHQQHSSNQGYEKASDIIHKFVNYEINNNVYSLHKFKSSNVNKNP  
KSEEEYKFLCNLYETHPEYSFEMLNDFKELSSKKIPYNDNCDEKDIKKVAHSRVFNHKNNNKRNKIVIRKYS  
DGNNIHTQIIIRGNGLNNNNNTSDDQDQNDYQYNNLDRMDDLFPNSIFSGLLKNFFIQNNNNNNNDGDGDYSSSEF  
DQNSSDDNQNDLFSELNNFFGFGNLADGMFGRKKKKKVLGFKTKEPQANLENNKENADD NIT QAIKQEKDVIIYL  
LNKVQKLNDINLKNFILQSLNNQNIRKILVDAVKKGYTNTYEQCKKEND NKS MYLLQMLKQSGIF

>PF3D7\_1025300 sent to GeneartAG

YETLNIRKSIDRKLTHLNNNIECAL NIA NYRNKKSKNLQFIGNNKIVKKCKNKYSNKVHLFGFSSKEEINNKGKKS  
LNIEKEYKNGNIFLYTKLFVNNEEKKFIIDLCSDHSFIFEKRLNEELLKCNNNNNNNNIFTQNDFEQKSPKNEGLNLQ  
YEKKDIENIKELFHL NNA QVDIKKVVNIKHEKEKQFPFYVINNEELDMNFGIIGFNFFKNYDFTVLDTIQMKILLN  
WNEGINTEDPNYDNIEYEKQKNGEQDQKEHEYYKINLYDYFNNIKYFNIIYVDNHLKYGIVDTGTNTILL NNA NIYNT  
TDDYNKDKTSTSIIESVLKNKYKV NKA NINHINIITKDNHKIPVELNKYIYSGNLEYINKDVILLGLDFLLNKKLIFDL  
KNNILIPKKYL NNA NEFNKKYDALNIGITNVQENQKSQNDNGNNHMDQIKIEDKCKEINYQLKAKELNFVRITKELE  
NDNVYMKDCKSTNDVIHKYAIKLLYGSNYLKKNDKNIEYEKRYNEVTEFFKKLNNEEKQNMNKIVNSVLLLR ND  
A IHDNNNNNNNNNNIINHQQHSSNQGYEKASDIIHKFVNYEINNNVYSLHKFKSSNVNKNPKSEEEYKFLCNLY  
ETHPEYSFEMLNDFKELSSKKIPYNDNCDEKDIKKVAHSRVFNHKNNNKRNKIVIRKYSDDGNNIHTQIIIRGN  
LNNNNNTSDDQDQNDYQYNNLDRMDDLFPNSIFSGLLKNFFIQNNNNNNNDGDGDYSSSEF  
DQNSSDDNQNDLFSELNNFFGFGNLADGMFGRKKKKKVLGFKTKEPQANLENNKENADD  
NIA QAIKQEKDVIIYLLNKVQKLNDINLKNFILQSLNNQNIRKILVDAVKKGYTNTYEQ  
CKKEND NKA MYLLQMLKQSGIF

16.

>PF3D7\_1137300 has 6 predicted TMDs (expressed the C-terminal)

MGLSLSSPQLPANAGSNITSNNNNNNHTNENQRVPFFQKIISIVQMLIMHMVMYFLSGGKNNMMSKSGIMDRNTNV  
LKNSFEKGDIFDIYFLSNNQSLDFEYLKNNSKLINIREKELYNHKTFSSEYKLNLYTFNLNDSWKDEKYSVVLVPIH  
YKKRNYPLIKNTTLKYEFKDKVLVENIPLTVKMVPFSEEEEEKYNLMDPSNNIKRNIEEQKKKKRNEQLYHIKRRID  
INIIYDDGKHMVKEFKMPYFKNWKNLTSNTYTPPIFLSDFWLEKDYVLLDDNFLKSKDKRVNYICTYDPYMLTKK  
QNDIHLTYTEQNMDENKKLHIEINYGTCSFIYFMFIKQISYSLNMMNETAASAATTTSTTTNNNNNNNNNNKIFS  
QNTNITANKEVHMKKILMTTNIYMLIFS AIFLLHSIFSFAFKNDMQFWYKNESMEGLSALS VITAFVCDIILALYL  
YDSEKTSWLLLFEMFIGVALSAWKVTKAVHVSFSKKYPFIIFKDKKNYTESMTKKYDKIAIKYVGILLIPCLIGYAIYA

LFYFKYKSWYSYIISVLAGTVYTFGFIMMTPQLYINYKLKSVEHLPWKALIKSLNTFIDDAFFLIDMPWMHKLSCFRDDIIFLCYIYQRCIYKVDKNRNETLTQNEKID NNNNAKQNDQLRELTQEQNVDVSHDKKND

>PF3D7\_1137300 has 6 predicted TMDs (expressed the C-terminal) sent to GeneartAG  
LYINYKLKSVEHLPWKALIKSLNTFIDDAFFLIDMPWMHKLSCFRDDIIFLCYIYQRCIYKVDKNRNEALTQNEKID  
NNNNAKQNDQLRELTQEQNVDVSHDKKND

17.

>PF3D7\_0830500 Tryp/Threonine rich antigen

MNLEQFKNINKDLATNLSQLSFLKNENKFLSQGKSLIKFLIGIAIFLVVLIETKSSHPALKEKKKKVLEFFENLVLNKK  
KKEKITAAIASKELADAETDTSDSEDEDHIINKKVRRKRNIINNPDEKVHNVKEKNSKSKNEEDKTDESYNETSLLS  
SDEGEVNLEDWKKNEWIKWMDTEEEWQLLKLWLEGEKNKWLEGNKEYDIWLNHMNSKWTNKNKDIDEEYD  
SNVFKDSYKWNEKQWEQWMKTEGKEFMLQDFKRWLEDSEGYLKSWLKQWQWKNMKILECLMNEWRRREEDEK  
WSNLEDTDQIRVLNHKDRKEWLRWKERVTRKLEWKHWVEMKENMNIYNKWKWKWKKNKLANFNEWSKNF  
IEKWIREKQWNNWINERKKYTSQRKSLEQQFGDNMDKMNKLKK  
KKILKFFPLFNYKSDLESIMEEDENEYNSFDENEEENDEKTDGVNVGKTEALNVAKTEGL  
NVGKTEDLNVAKTEDLNVAKTADLNAEKTDDLNSEKTADLNSDKTTDLNPEKTTNFNTYKATDLNANKAADLNSDKTTDLN  
SDKTTNFNTYKTDLYAEKTTDVNLGKTTNHNVAKTDDQKVVKHSLDHEVRQMI  
DQKVAQIMNHDLESTAEQKAEEKGGKAKAK TKVRTVDDDGNEINV

>PF3D7\_0830500 Tryp/Threonine rich antigen sent to GeneartAG

KSSHPALKEKKKKVLEFFENLVLNKKKKEKITAAIASKELADAETDTSDSEDEDHIINKKVRRKRNIINNPDEKVH  
NVKEKNSKSKNEEDKTDESYNEASLLSDEGEVNLEDWKKNEWIKWMDTEEEWQLLKLWLEGEKNKWLEGN  
KEYDIWLNHMNSKWTNKNKDIDEEYDSNVFKDSYKWNEKQWEQWMKTEGKEFMLQDFKRWLEDSEGYLKS  
WLKQWQWKNMKILECLMNEWRRREEDEKWSNLEDTDQIRVLNHKDRKEWLRWKERVTRKLEWKHWVEMKENMNI  
YNKWKWKWKKNKLANFNEWSKNFIEKWIREKQWNNWINERKKYTSQRKSLEQQFGDNMDKMNKLKKKILK  
FFPLFNYKSDLESIMEEDENEYNSFDENEEENDEKTDGVNVGKTEALNVAKTEGLNVGKTEDLNVAKTEDLNVAKT  
ADLNAEKTDDLNSEKTADLNSDKTTDLNPEKTTNFNTYKATDLNANKAADLNSDKTTDLNSDKTTNFNTYRTD  
DLN SDKTTNFNTYKTDLYAEKTTDVNLGKTTNHNVAKTDDQKVVKHSLDHEVRQMI  
DQKVAQIMNHDLESTAEQKAEEKGGKAKAK TKVRTVDDDGNEINV

18.

>PF3D7\_1462300: Conserved, unknown function

MNKLFLCFLVIIIYSLIYYCNDNILENANNDSTKDINNINKNSSNKSSEKTSNYNNDQYNIDDAYNEDEENDEEE  
GDDDENHFEVPINCSGGNCFDSMDINKIIDNKKKKMKYRRRRGKAIQIKPNVNHTELIIIEENLEIFKRIDKPIAIVS  
VLGDMHTGKSFLNLLNEHVIPDINKSNKYIENGFKVGNITASTYGIWVSEPIRINVKKLKKIYEYINENFTNFIKSY  
EYKDEYNEEIIDLLNLYKTDDWISNVEELNLSDDDEVNLLMDTQGLNSPNVKNRYDEILYALTNLISTDIIFLTMTKM  
INNKDLEFIEKITKANLMLRAYTRSSGSTFSIKNNFHKILDTLNENLLENSLILESIAKSNLMWVVHDFSQRDLVR  
KGGKLWLDILLNSDRRLDIKYWKIISNKSDDKHDAYTTKKQKIAYLTYNESSSGLLPNNNNNNNNNSNDS  
SNYKL  
NILYKNIDCVLLRNMYHNKEFDFTKANINDLNEEYKNDVLLRLRYKIYLRALLFPKKMYSDVQINNIINKIRKEQKLRE  
EQEKNKDNKKNDTNNQINDLNSNKDETSQSQTNRFMGHDVYDFITFLVKSANQNLFTNVNQFFKHFKINRAEISR  
NDLIFLYKKYLLQFMENEDVEFISNLNDENDDLFLQKEGNNTSVNHDENSADSLFNDHDENMQLKEHNKIDGEDN  
THQKSDSYKLPLLEIKKYEELIREQILNIWYKYTESDFHDEEEKELIKDIENNLIERLDQIKIEMLELGEMNIRKFCK  
IACEDALQIVVEDIKMKSQYPIKQKDLTTFFESVSYNLIKYLEKKLSKNSDKLDLIYKDNICSPLINNTFQEFYFLKK  
KNITLLENRLKSYFSNAVEKGKEVFEVLAENTEITEYFKNKNMFYTVLDKWTSEAINTYTLTSDFAKEEKEINDEY  
LNVLNNDIKLLQKAMEKWHNHCRDKTSALYNMHKSNLKKNFLENFNFLDELVLQDIFLNLKGQEELKYMDIYCANEDSWN  
SEYKNFLKLTDEIFQFIKIDENLKAQITCQQPLDELKNGIKGEIHKYYLWSSLKSTLYNRALIVLSSNIENIY  
IKNQRENKIQSNLDEKYNNKNKNYKRISKDLMSKVINRWLNNDIYNVYPIIRKQFLHRTVCYLGIAIFIISLVVYFK  
KFHASFLIILALFMVFGYQQISYIGKTFFRHVVFFYMYEGIANVFGTEGAIIVSILFISVTAIYVYNYHIVKFKKRVAKN  
TKKLLQSQNIMNMSGQNPYNDYNPSSKMRIFKPNFWRFDYDKDPKYHAPNIPHDYKMHNDSSQYMDLKNRSNRE  
NEMDDYTSFNSSKFQRRSYLD

>PF3D7\_1462300: Conserved, unknown function sent to GeneartAG

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LNEHVIPDINKSNKYIENGFKVGNITASTYGIWVSEPIRINVKKLKKIYEYINENEANFIKSYEYKDEYNEEIIDLLN  
LYKTDDWISNVEELNLADTDEVNLLMDTQGLNSPNVKNRYDEILYALTNLISTDIIFLTMTKMINNKDLEFIEKITKAN  
LMLRAYTRSSGSTFSIKNNFHKILDTLNENLLENSLILESIAKSNLMWVVHDFSQRDLVRKGGKLWLDILLNSDRR  
LDIKYWKIISNKA  
DDKHDAYTTKKQKIAYLTYNESASGLLPNNNNNNNNNSNDAS  
SNYKL  
NILYKNIDCVLLRN  
MYHNKEFDFTKANINDLNEEYKNDVLLRLRYKIYLRALLFPKKMYSDVQINNIINKIRKEQKLREEQEKNKDNKKNDT  
NNQANDLNSNKDETSQSQTNRFMGHDVYDFITFLVKSANQNLFTNVNQFFKHFKINRAEISRNDLIFLYKKYLLQF  
MENEDVEFISNLNDENDDLFLQKEGNNASVNHDENSADSLFNDHDENMQLKEHNKIDGEDNTHQKSDSYKLPLLE  
NEIKKYEELIREQILNIWYKYTESDFHDEEEKELIKDIENNLIERLDQIKIEMLELGEMNIRKFCKIACEDALQIVVEDIK  
MKSQYPIKQKDLTTFFESVSYNLIKYLEKKLSKNSDKLDLIYKDNICSPLINNTFQEFYFLKKKNIALNENRLKSYF  
SNAVEKGKEVFEVLAENTEITAEYFKNKNMFYTVLDKWTSEAINTYTLTSDFAKEEKEINDEYLNVLNNDIKLLQK  
AMEKWHNHCRDKTSALYNMHKSNLKKNFLENFNFLDELVLQDIFLNLKGQEELKYMDIYCANEDSWNSEYKNF  
LKLTDIEIFQFIKIDENLKAQITCQQPLDELKNGIKGEIHKYYLWSSLKSTLYNRALIVLSSNIENIYIKNQRENKIQSNL  
DEKYNNKNKNYKRISKDLMSKVINRWLNNDIYNVYPIIRKQF

**>PF3D7\_0317100: 6-CYS (B9)**

**>PF3D7\_0317100: 6-CYS (B9) sent to GenentAG**

**>PF3D7\_0823800: DnaJ protein, Putative**

**>PF3D7\_0823800: DnaJ protein, Putative sent to GeneartAG**

**>PF3D7\_1229300 Conserved plasmodium protein**

335

KYEDYIKSLPEAFHLAKGDSKKDEMCKENPKEENDNNNDNKA<sup>NSNSNNNYKVGMQDIVN</sup>NIANKISDDNKNKNDNN  
 ASDKYDCNDDDDNNNTNSLSL<sup>FHKMMC</sup>AFKKKLFKNEQVIEKDIRNVKKGVINLKENIVKDS<sup>SNIA</sup>KHTNLKDDITKN  
 TNYL<sup>FNLF</sup>FKSKFEEKGDVNVK<sup>SSDY</sup>FNKLNLPDSL<sup>HLFFSKLL</sup>TL<sup>SQND</sup>DKDKNKITHIN<sup>YYNN</sup>KMNDNTENN  
 ILLQNL<sup>LSFK</sup>FNK<sup>QK</sup>DEYNN<sup>DN</sup>NK<sup>EN</sup>DEFF<sup>HSFY</sup>LHNENDKHKNKDKKY<sup>YSW</sup>WFTNKNDEKKNVDNNLL<sup>LYN</sup>  
 NANN<sup>SN</sup>STFN<sup>Q</sup>QA<sup>DHEN</sup>LPEQVREK<sup>NS</sup>QEEKNK  
 WLDIFW<sup>KY</sup>FHKNEEEIEKKEMNEKNDLNDNNKNGVNV<sup>DDHNL</sup>GLHNLDDKKNK<sup>FS</sup>FLNY  
 WNKDKDIPKEGSADEYSQDNNNNKA<sup>DDQNN</sup>NDKKFNID<sup>MY</sup>FGKNK<sup>KN</sup>DEMDMKNK<sup>EM</sup>MDM<sup>DN</sup>KDM<sup>KN</sup>KD  
 MNDKNDNM<sup>DM</sup>MNNMNNMNNM<sup>NG</sup>DDTN<sup>VD</sup>SDTLN<sup>NL</sup>KNEM<sup>NK</sup>VDD<sup>DP</sup>KGFVLNLV<sup>KNY</sup>EEN<sup>NK</sup>N  
 VDNINYSLL<sup>TS</sup>GKETNENIN<sup>YH</sup>PLIK<sup>FK</sup>SCLM<sup>NC</sup>FK<sup>EN</sup>NT<sup>EK</sup>SV<sup>ES</sup>YLS<sup>FD</sup>DDYKILEK<sup>CS</sup>CK<sup>KN</sup>N<sup>NL</sup>ND<sup>AH</sup>NED  
 PPMKKDD<sup>TI</sup>LYMEEK<sup>NK</sup>IND<sup>TT</sup>KNLL<sup>ND</sup>NIN<sup>LN</sup>EN<sup>NE</sup>STEK<sup>TS</sup>SK<sup>WS</sup>DD<sup>FF</sup>FK<sup>NN</sup>K<sup>KN</sup>IK<sup>DS</sup>YEED<sup>KNS</sup>NN<sup>PQ</sup>  
 D<sup>NI</sup>AA<sup>Q</sup>TSNETNLEN<sup>IL</sup>ND<sup>NL</sup>STHLMYDINN<sup>NN</sup>KK<sup>NS</sup>NN<sup>KN</sup>KN<sup>IS</sup>NN<sup>NY</sup>KMK<sup>N</sup>FL<sup>DS</sup>SK<sup>FK</sup>YS<sup>NE</sup>KE<sup>TK</sup>N  
 FIQDEQ<sup>LD</sup>DKN<sup>VN</sup>Y<sup>NK</sup>PL<sup>HF</sup>FN<sup>NS</sup>YN<sup>TD</sup>DDNNNNNNNNNN<sup>NY</sup>K  
 DN<sup>LM</sup>NA<sup>SV</sup>NDNLLN<sup>DD</sup>KY<sup>NS</sup>LKN<sup>NN</sup>NNNN<sup>NIM</sup>LKN<sup>DI</sup>DND<sup>EN</sup>NN<sup>YIS</sup>

MEKTSFKNNQKSYSKRNSSLKKKVNTDKNDNSLLTIYSFISNYMLKEEMNYDENDNRYILLESQDIENECGLKKVSF  
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RQLWKKKKKKKKRKEKMMYTNSNSKEYKGNLAKYFTLSMDNNGKEENIKFDEKQLSRQMSYYNDAYTYTSE  
MIYKNNRRNKEKFALYLRMSLFNIIGLYLYEYPHTNNDMDTFDGTFFKNMHKIDREDIKDIISKNEINKDIDYHNYMK  
KYNLNVSIILNKQKEFFENKSIIGNGKASSSSNFSNDNNNNNNNNNNNNNNSMHVNTFDRSINNEDSLINTNNLNNDN  
NNFLRFLRNIFSRNNYEQEDNLKSSSNLINKEKTNNTNTVTNTGYNNLYKNNNNNNNIIDVEKNIYEEKDKNNNLKK  
KNFKNVKNNNNYNSGVYYVNDKMDLKDIKTEDVNLLIYMIDILNLMDDEHIMTYIYNEMNKIYKAIYSFFGELLY  
SIIVFSFLILKILKIDNNKFSVILTMYGIFYKIFYFAHYVLFMGLYLSIYNIYIHRDILTHSYNFFCYHYVYNNLYIHL  
LMLFYALQNVITFDANFCIRMVFILIKVHIKIYKIKTNFTFYELKEDKSPSLSTCFNKLKNKCNMNCKCCKDKN  
CVDIDIDEMHDIDYEKNKLPFPNPNRPYCKNLNIKIYEQSLLNMRNPFIK

PHTNNDMDTFDGTfKNMHIKDREDIKDIISKNLKDYHNMYMKKVN**L****NVA**ILNKQKEFFE**NKA**IGNGKASS**S****NFA**  
SSN  
DNNNNNNNNNNNSMHVNTFDRSINNEDSLINTNNLNNDNNFLRFLRNIFSRNNYEQEDNLKSSSNLINKEKT**N**  
**NANVA**NTGYYNLYKNNNNNNIIDVEKNYYEYKDKNNNLKKNFKNVKNNNYNSGVYYVNDKMDLKDIKTEDV  
NNLIYMIDLNNLMDDEHIMTYIYNEMNKIYK

MNKGLSKILELGGFFVGSFFHIWYITG EKIFKRKKKIVKENILLRLNLCYEIYTVLCETSKTTKHVYDMLK NESNTI IELS  
RLEEVLNNGYKKKIEDVEKNVLKRF NVSVEDFYEELKNYEKDPDVQKYLSIKKMYNEALLGLQPKLPSIDKISE  
DVILNTLSIYIEKKRKIYTKIDMSMLKNNEIIDIQNTLSNAQFFENLHKSTQHVEEQIKENNLVNLPLTFKYLVSYSN  
DPNFLQKKKYLESKHGEKMIKILKIKTSKKKKRAHKDEQRSS NSSVSFKTSPSLDVYNVPHLGLTEKEEDHVSYSQS  
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SGIYEEEEHASGIYEEDHEERYTSQVRSERSIKSVTSQQDDIIHSHEEDNYVSREVIHDDSFVGDKDKQIFDDNYVSER  
EEIKQDDDYISEREIKQDDDYISEREIKQDDDYISEREIEFKQDESFGDKQIFEDDDYVSEREIKQDDDYISEREIE  
KQDDDYVNEREIEIKQDDDYVSEREIEKQDESFGDKQIFEDDDYVSEREESKQDESFGDKQIFEDDDYVSEREIE  
KQDDDYVSEREIEKQDESFGDKQIFEDDDYVSEREIEFKQDESFGDKQDNFDDDYVSEREDIKQDDDYISERDEI  
KQDDSFAGDKQDNFEDDDYVSEKEI IYDNN NISDREQVIQDDNYVSDKEI IYDNNNNN NISDSEVDKENNNY IYE  
NDEI IK NKS MNEKDSIIHYDEQTVKNKDIIENVKEENSIDQMLENDIINNINNEIIDNNENIIN NOSVENNTSMTE  
YEDVIDMDYKSPENLFMFQDEYNINEDHKGEINNNDNNSKSVSGMGEREVSNEENREDEMRNEEVDYIKDDDEH  
NTDDIKEDDEHNADDIKEDDEHNVDDIKEDDENNADDIKEDDEHNADDIKEDDEHNTDDIKEDDEHNTDDIKEDDE  
HN ADDIKEDDENNADDIKEDDEHNTDDIKEDDEHNTDDIKEDDEHNADDIKDDEHNTDDIKD  
DEHNTDDIKDDEHNADDIKEDDNDQELNENLEGS DVPKDDEALNIEDTSEEQKETDDKIESGSRDKKNEGS  
TEKKIETKKGGLLMGIMKRKRFRMSRDPKKKS

EKIFKRKKKIVKENILLRLNLCYEIYTVLCETSKTTKHVYDMLK**NEANTA**IELSRLEEVLLNNGYKKKIBDEVKNVLK  
 RF**NVA**VEDFYEELKNYEKDPDVQKYLNSIKKMYNEALLGLQPKLPSIDEKISEDVIIN**LA**SLIYKEKRKIYTKIDSML  
 KNEIIDIQNTLSQAFQFFENLHKSTQHVEEQIKENNVLNPLTFTFKYLVSYYSNDPNFLQKKKYLESKHGKEMIKILK  
 KTSKKKKKRAHKEDRQSS**NSA**VSFKSTPSLDYVNVNPHLGLTEKEEDHYSQSKSEMEHMDKSDHVDIFPHNVANDL  
 KRDEQIEYISESGDMQPEKYYEGEHITIRYEEEEEDISEIYEEDRASRIYEEDRASGIYEEEHASGIYEEDHEERYRTSQV  
 SERSIKKSVTSQQDDIIHSHEEDNYVSDREVIHDDSFVGDKDQIFDDNYVVSEREIHKQDDDYISEREIHKQDDDYISERE  
 EIKQDDDYISEREFEKQDESFGDKDQIFEDDNYVVSEREIHKQDDDYISEREIHKQDDDYISEREIHKQDDDYISERE  
 IKQDESFGDKDQIFEDDNYVVSEREESKQDESFGDKDQVFDDDYVSEREIHKQDDDYVSEREIHKQDESFGDKDQI  
 FEDDNYVSEREFEKQDESFGDKDQNFDDDYVSEREDIKQDDDYISEREIHKQDDDSFAGDKDQNFDDNYVSEKEEI  
 YDNN**NIA**DREQVIQDDNYVSDKEEIIYDNNNN**NIA**DSEVDKENNNYVYENDEIHK**NKA**MNEKDSSIHYYDEEGTVK  
 NKDIENVKEENSIDIQMLENDIINNENIIDDNNENIIN**NQA**KVENNTSMTEYEDVIDMDYKSPENLFMFDDQEYNINE  
 DHKGEINNNDNDSKSVSGMGEREVSNEENREDEMIRNEEVYIKDDDEHNTDDIKEDDEHNADDIKEDDEHNVD  
 IKEDDEHNADDIKEDDEHNADDIKEDDEHNNTDDIKEDDEHNTDDIKEDDEHNADDIKEDDEHNADDIKEDDEHNNTD



DIKEDDEHNTDDIKEDDEHNADDIKDDEHNTDDIKDDEHNTDDIKDDEHNADDIKDEDNDQVEDNQELNENLEGSD  
VPKDDEALNIEDTSEEQKETDDKIESGSVRDKKNEGSTEKKIETKKGGLLMGIMKRKRFRMSRDPKKKS

24.

>PF3D7\_1252300

MSDPWADYDPNIISALQEAFGDIITAKPLTNLLSGESGPAIAGGCSCNRSLMIFIALMVFNAILFWVYNRGKKDIEKKI  
KKHGY

>PF3D7\_1252300 sent to GeneartAG

SDPWADYDPNIISALQEAFGDIITAKPLTNLLSGESGPAIAGGCSCNRSL

25. A&B

>PF3D7\_0629500 AMINO ACID TRANSPORTER, PUTATIVE

MNKKYGTSSNNHDNKKDKKNNADKNKKNKNTTTGEENKDSNKS LVNDSKKNDSKKNKYNIVKANIKNIFASDK  
KNEKSDKNEKNESKSSKNTETYTNVNDKKSNNLITKGSNDK KKKKKKDSKKNSSNNNNNNNTIVDISDGDYTNDEEG  
TNKPKRNWKGRTFSRFTPGGVRSSSTVLFICTAIGVGFLSIPYVFSKLGIIISIIILNAFESYVTTNICTSSLEHNTFVYG  
NLLKKIGNKYKTIIDFGLSFGFVSSYILILISNFLTIFYVFNFPTLFTNNVFLVILICLLIPITFRNKVGSLNHFLIFS  
FSLTITVLTIGLQTKSYNNLLINKEVNLFKMDKHFFKCFNILLFSFSQQPNA CFITGQFNQPTHRRLNKSTFRSVILQVIF  
YTLFGILGYFSFLNTAKDNIVLNYENS NVSILLCFLLSLTFFFSVPLNEMGSYQSMALGITTRDALYKLYTYIFRRTG  
YSANLSLLLSEYTNDPYQETHADNITEHSSVSESQTDDQNQRMWISVIVTIFCALIACKVKKLSNVIGIGGGITSTLISC  
LLPNLIYYKNRHNVS NKLKRYSTLFMLCFFSFMGFLSVVVT TLNLIL

>PF3D7\_0629500\_SEG1 AMINO ACID TRANSPORTER, PUTATIVE sent to GeneartAG

QTKSYNNLLINKEVNLFKMDKHFFKCFNILLFSFSQQPNA CFITGQFNQPTHRRLNKATFR

>PF3D7\_0629500\_SEG2 AMINO ACID TRANSPORTER, PUTATIVE sent to GeneartAG

GSYQSMALGITTRDALYKLYTYIFRRTGYSANLSLLLSEYTNDPYQETHADNIAEHSSVSESQTDDQNQRMWISVIV  
TIFCALIACKVKKLSN

26.

> PF3D7\_1345100 THIOREDOXIN 2 (TRX2)

MKKYIFFFLFSFINFFVYDVTCTKEVTSTND DPLTPLNRFDKY YLRMFKKVPRLQQNGSNIINGVNMKNTVIVLYFF  
AKWCQACTMQSTEMDKLQKY YGKRIYLLKVLDK NESLARKF  
SVKSLPTIILLKNKTM LARKDHFVSSNDLIALIKHL

> PF3D7\_1345100 THIOREDOXIN 2 (TRX2) sent to GeneartAG

TKEVTSTND DPLTPLNRFDKY YLRMFKKVPRLQQNGANIINGVNMKNTVIVLYFFAKWCQACTMQSTEMDKLQKY  
YGKRIYLLKVLDK NESLARKFSVKSLPTIILLKNKTM LARKDHFVSSNDLIALIKHL

27.

>PF3D7\_0206200 Pantothenate transporter (PAT)

MAKNQYMEDRNIREPNTLLGEETEQLVDSFHYENNSSSIYKKVNSNRSKNGKHSMAFHKSLAVVNVAAGLDGCDD  
QLLPASFRAL EADLN LHPSLLGYITLAQTLMLSLFSPIWGFLSDKYSRKWMLVFGTALWGVATILLANINDFAHILFFR  
AINGLALGSIGPISQSILADA AKNESLGLSFGLYQLSSSLGR LIGGVVTTTVALKYFGGIRGWRLCFIVVGILSVLLSIIV  
ALFVEDAPRQVRKNKMDYLDGESNTNASNNNNNSNNNNINNNINMNSLDNNNSFTGLSHQSTRTYILYQNIVEL  
LKDSLSKKSHIILLEGFTGTIPWLA LSFNTMFFQYCGLSDLQAAIITGFL LIGSAIGGVVGGHFGDIMHDISNKHGRPLI  
GQLAMFGRVPLVLLIYLVIPKRKESFELFALSCFCIGLSSIA GVA VNRPIVSDIIRPDYRGTVFSLTIAIEGVGSSLIGAPL  
FGYLA EKIFYQNNNLLISDMPEDIRINNAQALS KTLFYLTII PWILSFIFYSL LHFTYGKEYLKM  
NEIIQNEYKYDDEDEETIPEKKMLT

>PF3D7\_0206200 Pantothenate transporter (PAT) sent to GeneartAG

DAPRQVRKNKMDYLDGESNTNAA NNNNNNSNNNNINNNINMNNALDNNNSFTGLSHQSTR  
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## Appendix 5.1. Supplementary information of Nyamisati cohort and optimisation of antibody detection by Luminex and indirect ELISA

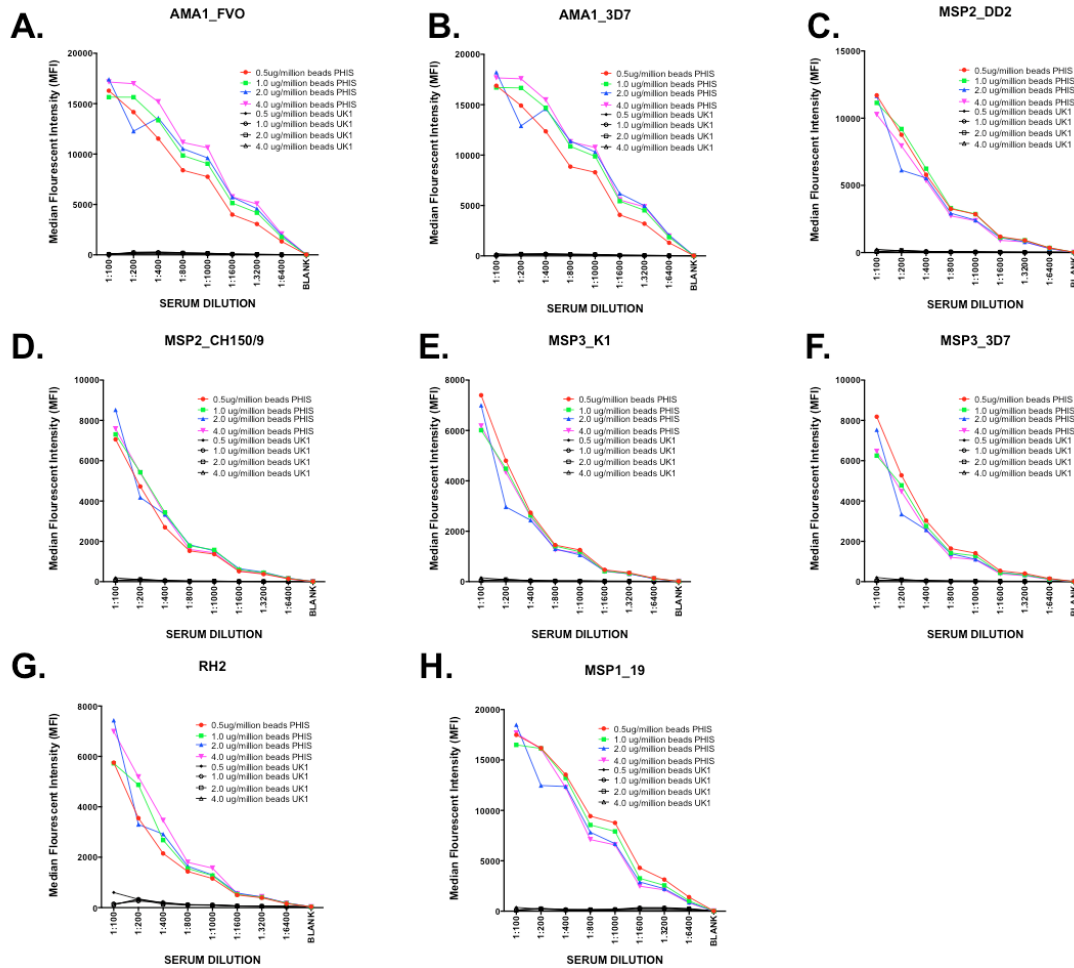


Figure 1: Optimal antigen and antibody concentration estimated by checkerboard titrations for Luminex assays. A checkerboard titration using varying concentrations of antigen and serum dilution was performed for each antigen to determine the optimal concentration for use in the larger study. A pool of sera from adults (PHIS) was used as a positive control and sera from non-exposed UK adults was used as a negative control. As shown in A & B, antigen saturation was achieved at 1.0µg/million beads for AMA1 (3D7 and FVO allele) and 0.5 µg/million beads for the other antigens namely MSP1-19, MSP2 (DD2 and CH150/9 allele), MSP3 (3D7 and K1 allele) and RH2-2030 (C-H). There was low reactivity observed with the negative control sera with median fluorescent intensity (MFI) values of less than 500.

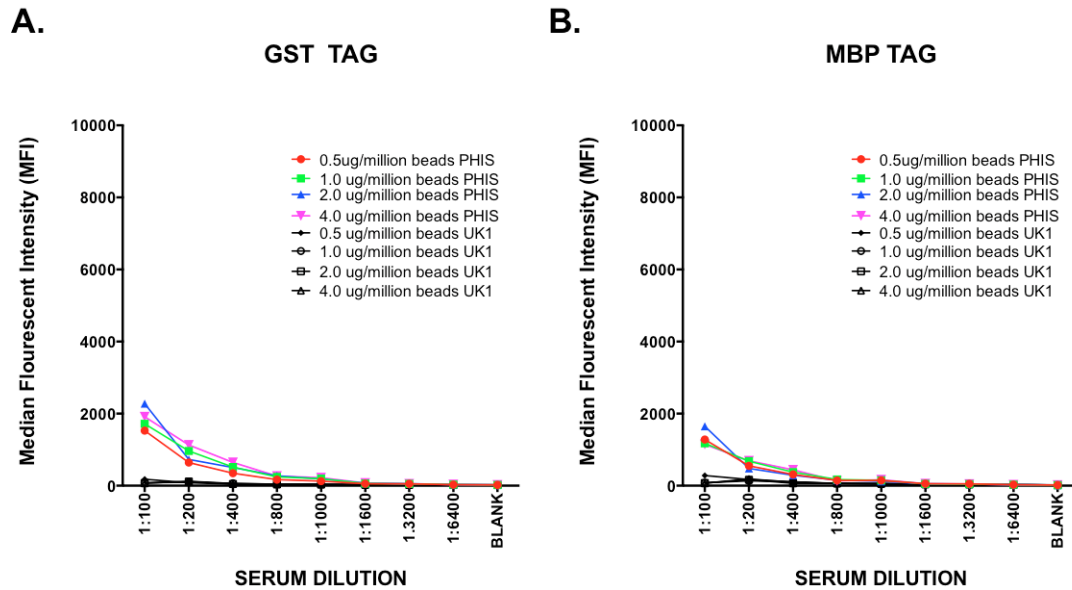


Figure 2: Optimal antigen and antibody concentration estimated by checkerboard titrations for Luminex assays. A pool of sera from adults (PHIS) was used as a positive control and sera from non-exposed UK adults was used as a negative control. The GST (A) and MBP (B) tags had low reactivity with PHIS of less than 2500 MFI values at the highest sera dilution tested.



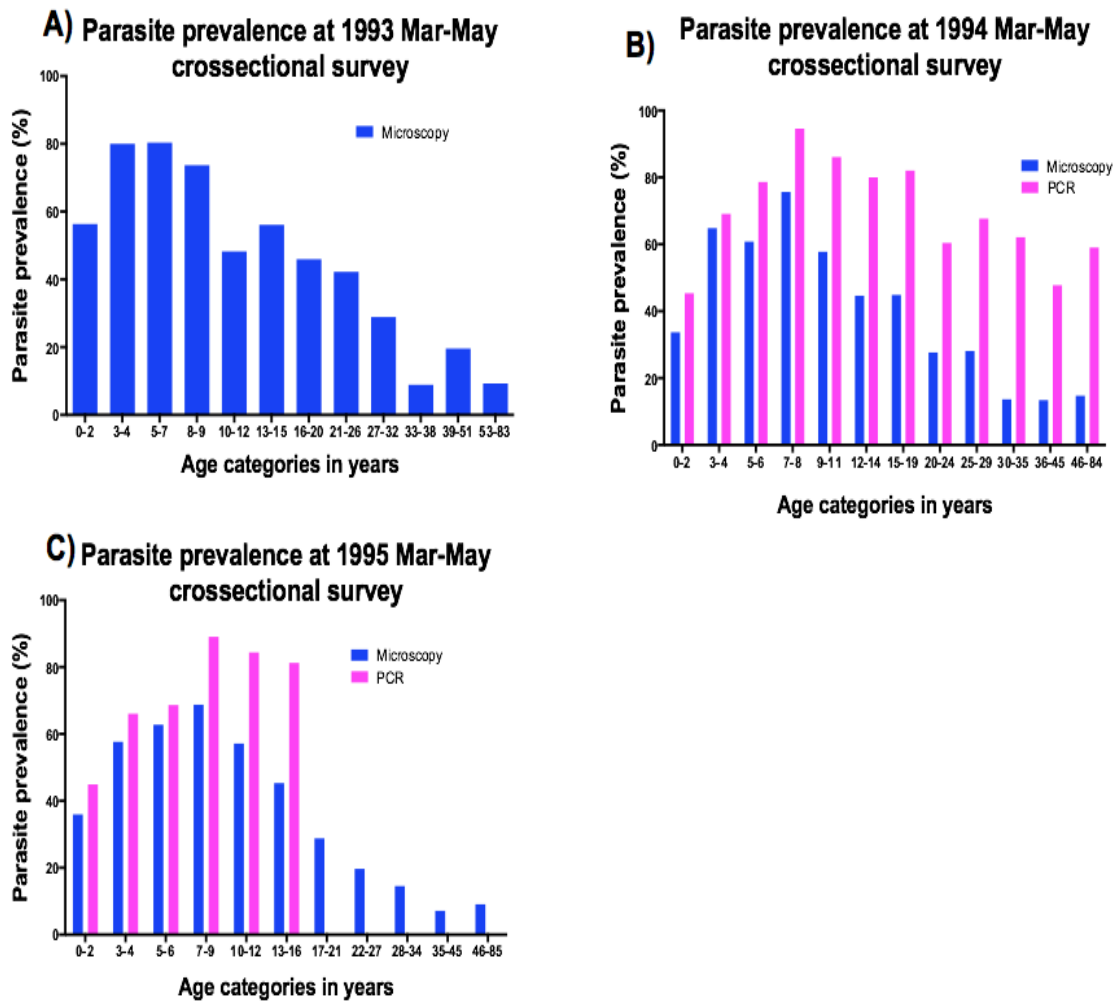


Figure 3: Significantly higher PCR parasite prevalence in older children and adults. Figure showing the parasite prevalence by age in the entire cohort using data collected at A) 1993 B) 1994 and C) 1995 cross-sectional survey. Blue bars show parasite prevalence determined by microscopy. Purple bars represent parasite prevalence determined by PCR. PCR data is not available for the 1993 survey and for the over 16 year olds in the 1995 survey. The parasite prevalence based on microscopy rose with age reaching a peak of between 68 and 80% at about seven years of age, and then declined gradually thereafter. The lowest parasite prevalence by microscopy ranged between 7 and 13% and was observed in older adults aged 30 to 85 years. Parasite prevalence estimated by PCR data showed overall higher parasite prevalence in all age groups when compared to microscopy. This was particularly striking for the older age groups (over 6 years olds) as seen in the 1994 (B) and 1995 (C) data. For example, using data from the 1994 survey in adults aged between 30-35 years, microscopy

underestimated the prevalence of asymptomatic parasitaemia by 48% when compared to the prevalence estimated by PCR data.

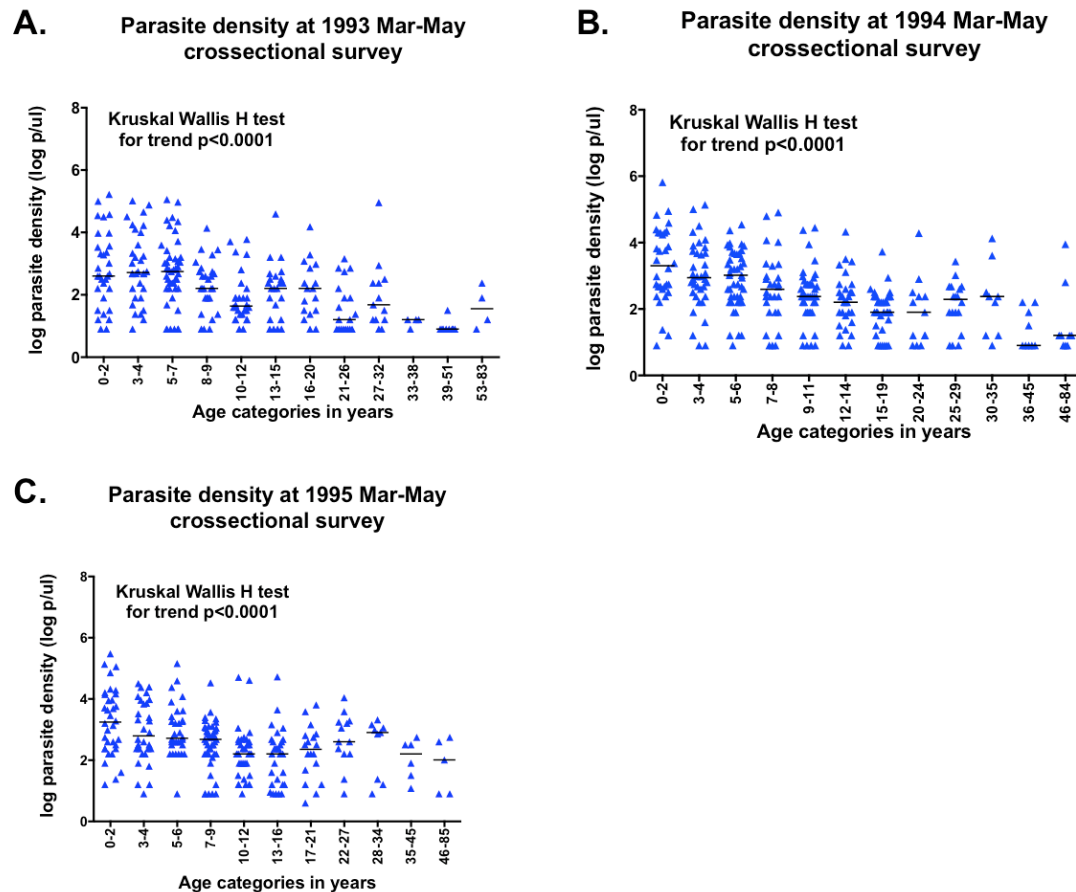


Figure 4: Parasite densities decline significantly with age in all cross-sectional surveys. Figure shows the  $\log_{10}$  parasite density by age in the entire cohort using data collected at the A) 1993 B) 1994 and C) 1995 cross-sectional survey. The Kruskal-Wallis H test for trend was used to compare media parasite density levels with age. The median parasite density dropped gradually with age in the entire cohort. This decline was statistically significant for all the surveys. The highest median parasite densities were recorded in children aged 5-7 years in the 1993 survey (560 parasites/ $\mu$ l, range 8-114,025) and in children aged 0-2 years in the 1994 and 1995 surveys (2009 parasites/ $\mu$ l: range 8-660,693 and 1754 parasites/ $\mu$ l: 16-300,000 in the 1994 and 1995 surveys respectively). The lowest median parasite densities recorded were 8 (range: 8-32), 8 (range: 8-160) and 100 (range: 8-560) parasites/ $\mu$ l in adults aged 39-51, 36-45 and 46-85 years in the three consecutive surveys.

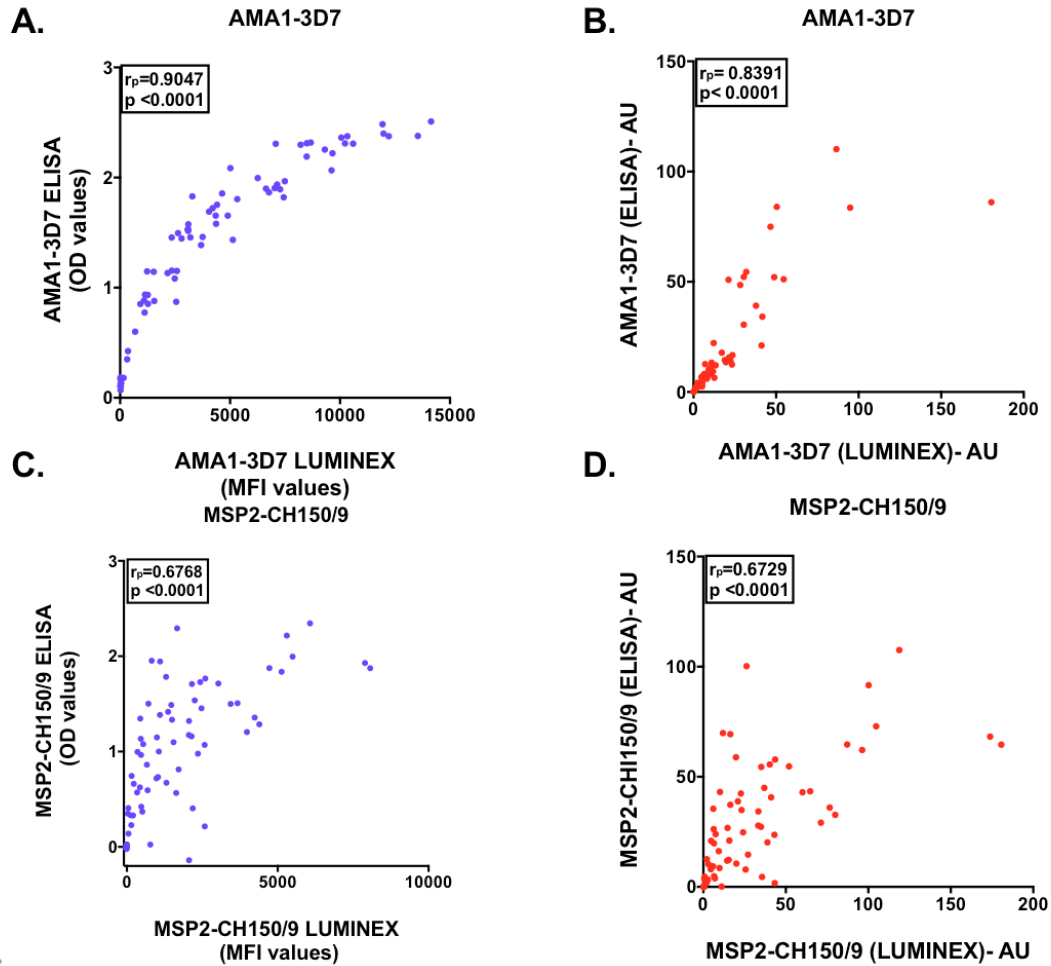


Figure 5: High correlations between responses measured using different platforms. Correlation between antibody responses as determined by ELISA and Luminex assays using 72 serum samples for AMA1 (A and B) and MSP2 (C and D). Blue graphs represent correlation between optical density and median fluorescent intensity values (MFI) obtained following ELISA and Luminex assays respectively. Red graphs represent extrapolated antibody concentrations (AU) from a standard curve using a pool of purified immunoglobulins from malaria-immune IgG (MIG) of known concentration.

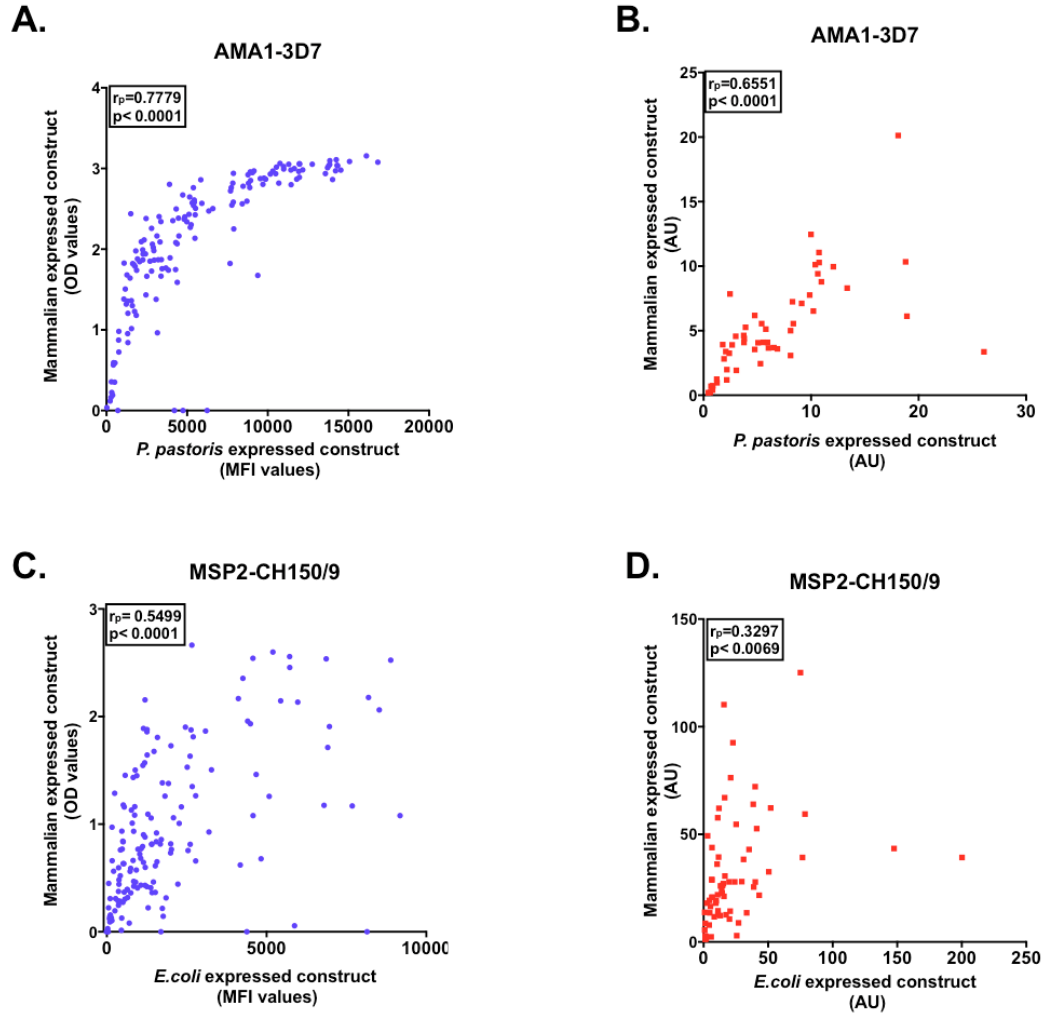


Figure 6: Correlations between responses measured in antigens expressed using different heterologous expression systems vary. Correlation between antibody responses as determined by ELISA and luminex assays using 72 serum samples for AMA1 (A and B) and MSP2 (C and D) obtained using different expression system. Blue graphs represent correlation between optical density and median fluorescent intensity values (MFI) obtained following ELISA and luminex assays respectively. Red graphs represent extrapolated antibody concentrations (AU) from a standard curve using a pool of purified immunoglobulins from malaria-immune IgG (MIG) of known concentration. Two constructs of AMA1 were tested, one expressed in *Pichia pastoris* and a second expressed using mammalian cells. Similarly, two constructs of MSP2 were tested, one expressed in *Escherichia coli* and a second expressed using mammalian cells.

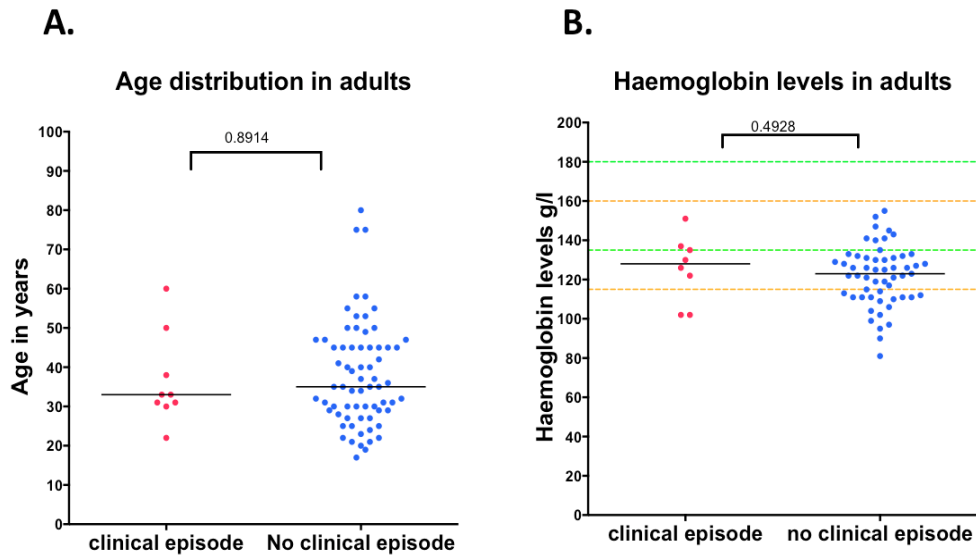


Figure 7: A comparison of the age and haemoglobin levels between the adults who experienced a febrile malaria episode compared to those who did not. A) The median age in years in adults who had a clinical episode versus those who did not was 33 versus 35 years: p-value=0.8914. B) The median haemoglobin levels in adults who had a clinical episode versus those who did not was 128 versus 123 g/l: p-value=0.4928. Yellow lines indicate the normal haemoglobin levels in adult females while the green lines indicate normal levels in adult males.

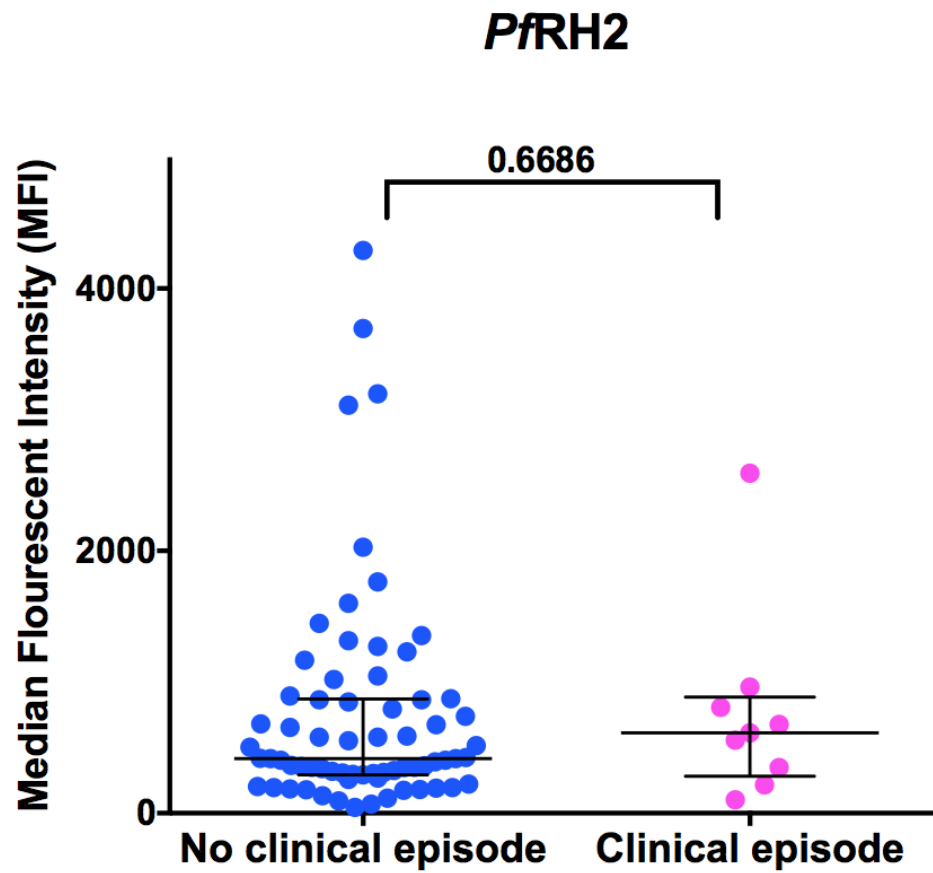
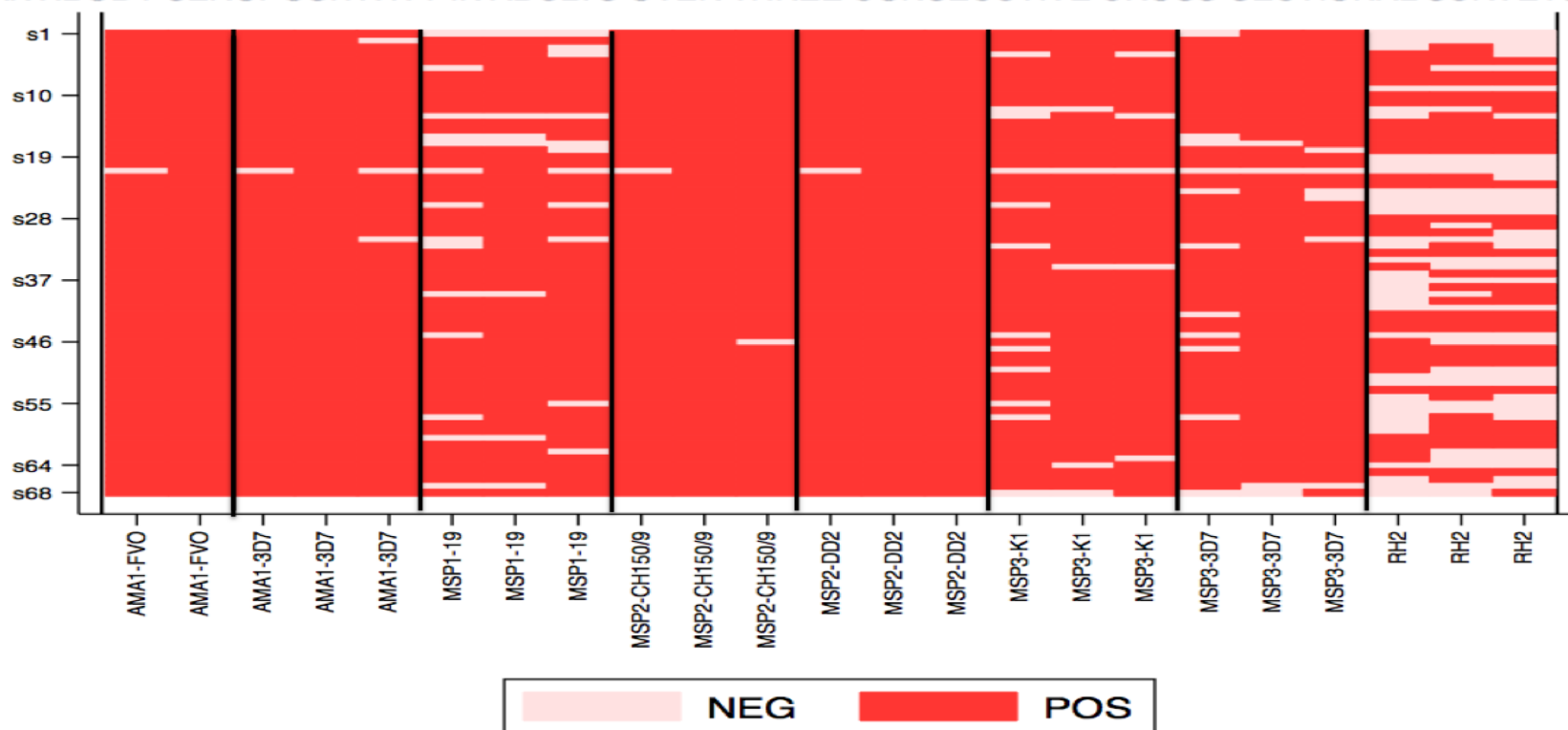


Figure 8: Antibody levels to *Pf*RH2, the only antigen among those tested in which antibody levels were higher in individuals who experienced a clinical episode compared to those who did not.

# ANTIBODY SEROPOSITIVITY IN ADULTS OVER THREE CONSECUTIVE CROSS-SECTIONAL SURVEYS



**Figure 9: The majority of adults have stable responses to the well-studied antigens over a 3 year period.** Plot matrix showing the seropositive response to AMA1 (FVO and 3D7), MSP1-19, MSP2 (CH150/9 and DD2), MSP3 (K1 and 3D7), in 68 clinically immune individuals with samples collected at the 1993, 1994 and 1995 cross-sectional survey. Each row on the Y-axis represents an individual while the X-axis shows responses to each antigen grouped together and arranged in consecutive years. For example, for AMA1-3D7, the three consecutive columns represent 1993,1994 and 1995 responses. Antibody measurements to AMA1-FVO were measured at the 1993 and 1994 survey only.

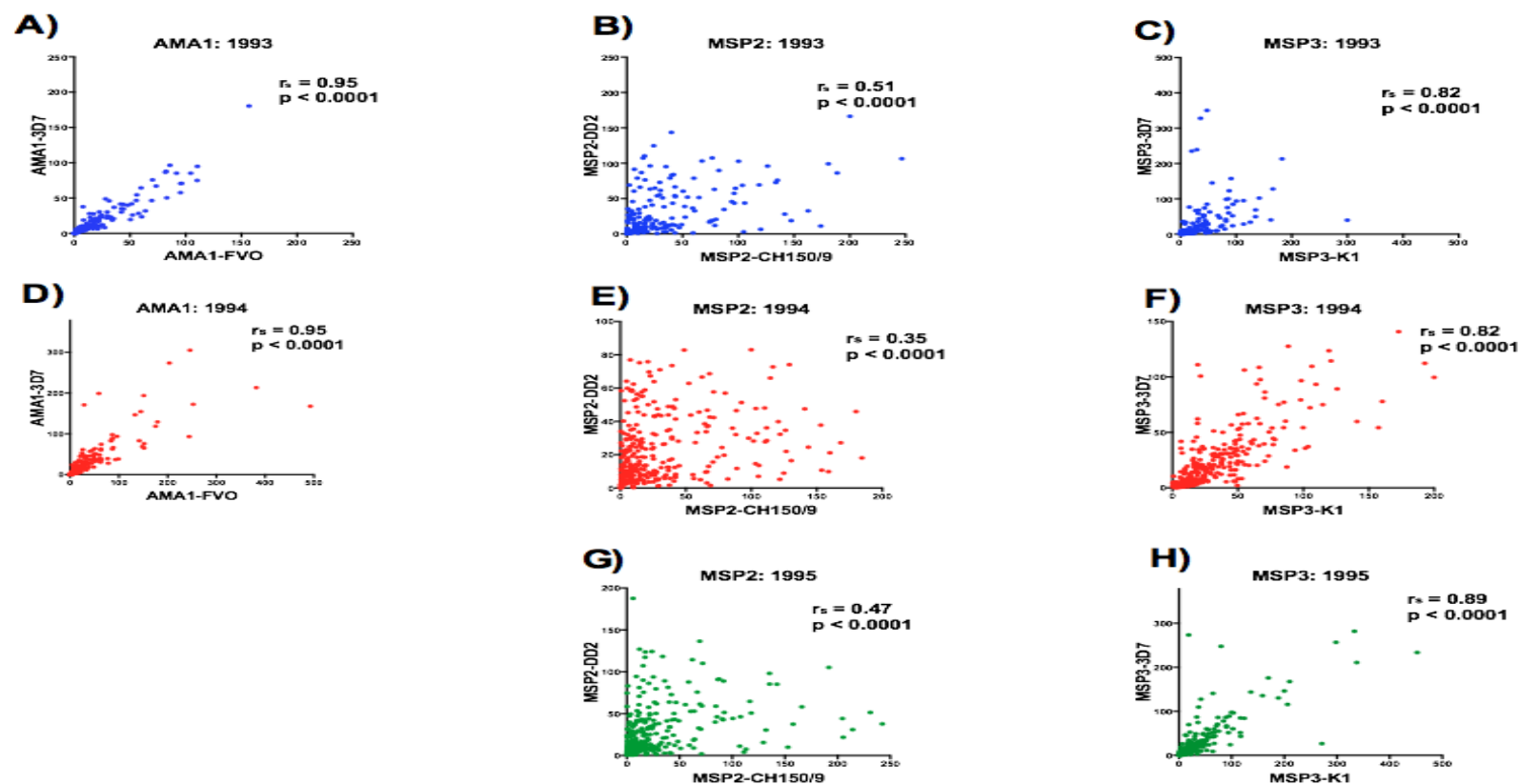


Figure 10: High correlations between two alleles of the same antigen suggesting potential cross-reactive AMA1 and MSP3 responses in adults. Scatter plots showing correlation between antibody responses to AMA1-3D7 and AMA1-FVO alleles, MSP2-DD2 and MSP2-CH150/9 alleles and MSP3-3D7 and MSP3-K1 allele using sera from semi-immune adults collected at the 1993, 1994 and 1995 cross-sectional surveys. Antibody measurements for AMA1-FVO were not measured at the 1995 survey. Antibody responses to AMA1-3D7 and FVO alleles were highly positively correlated with Spearman correlation coefficient of 0.95



at both the 1993 and 1994 cross-sectional survey (figure 18A and 18D). Similarly, antibody responses to MSP3-K1 and 3D7 alleles were also highly positively correlated with a Spearman correlation co-efficient of 0.82 at the 1993 (figure 18C) and 1994 (figure 18F) survey and 0.89 at the 1995 cross-sectional survey (figure 18E). Antibody responses to MSP2-DD2 and MSP2-CH150/9 were positively but less strongly correlated with R-values of 0.51 (figure 18B), 0.35 (figure 18E) and 0.47 (figure 18G) at the 1993,1994 and 1995 cross-sectional survey. This suggests that responses to AMA1 and MSP3 are potentially cross-reactive but doesn't exclude an allele specific component that could be confirmed by competition ELISAs, while MSP2 induced a more obvious allele-specific response.

## Appendix 5.2. Comparison of antibody breadth score between individuals who experienced a clinical episode versus those who did not.

Appendix 5.2 shows breadth scores in individuals who experienced a clinical episode of malaria. The breadth of antibody responses to known and novel antigens in 77 adults in the Nyamisati cohort. Black squares indicate a response was detected while white squares indicate a negative response to a specific antigen in the respective individual. Yellow highlights the individuals who experienced a clinical episode over the three year period.

Age	Clinical episode	PF3D7_0823800	PF3D7_1252300	PF3D7_1306000	PF3D7_0206200	PF3D7_0830500	PF3D7_1345100	PF3D7_0629500[SEG2]	PF3D7_0738002	PF3D7_0925900	PF3D7_1025300	PF3D7_1401600	AMA1	MSP2	RH2	MSP1-19	MSP3	Breadth score
50	No																	7
36	No																	16
35	No																	6
49	No																	5
55	No																	13
34	No																	7
45	No																	13
31	No																	8
32	No																	7
45	No																	14
53	No																	4
31	No																	14
29	No																	5
21	No																	11
45	No																	4
40	No																	12
35	No																	10
45	No																	15
41	No																	15
58	No																	14
40	No																	12
47	No																	9
25	No																	8
75	No																	14
24	No																	4
34	No																	8
40	No																	8
50	No																	9
31	No																	14
55	No																	3
27	No																	6
28	No																	12
53	No																	14
35	No																	8
19	No																	12
45	No																	10
45	No																	8
21	No																	5
50	No																	3
30	No																	10
47	No																	11
42	No																	7
17	No																	12
23	No																	15
25	No																	7
29	No																	2
37	No																	5
47	No																	12
25	No																	12
27	No																	10
27	No																	3
75	No																	13
80	No																	12
22	No																	11
39	No																	12
20	No																	10
29	No																	15
30	No																	11
37	No																	6
58	No																	9
30	No																	16
30	No																	8
22	No																	4
32	No																	5
35	No																	2
45	No																	13
30	Yes																	11
60	Yes																	4
31	Yes																	6
33	Yes																	3
31	Yes																	16
22	Yes																	7
50	Yes																	10
30	Yes																	5
45	Yes																	8
38	Yes																	3
33	Yes																	2

## Appendix 6.1. Supplementary data on immune effector mechanism in adults

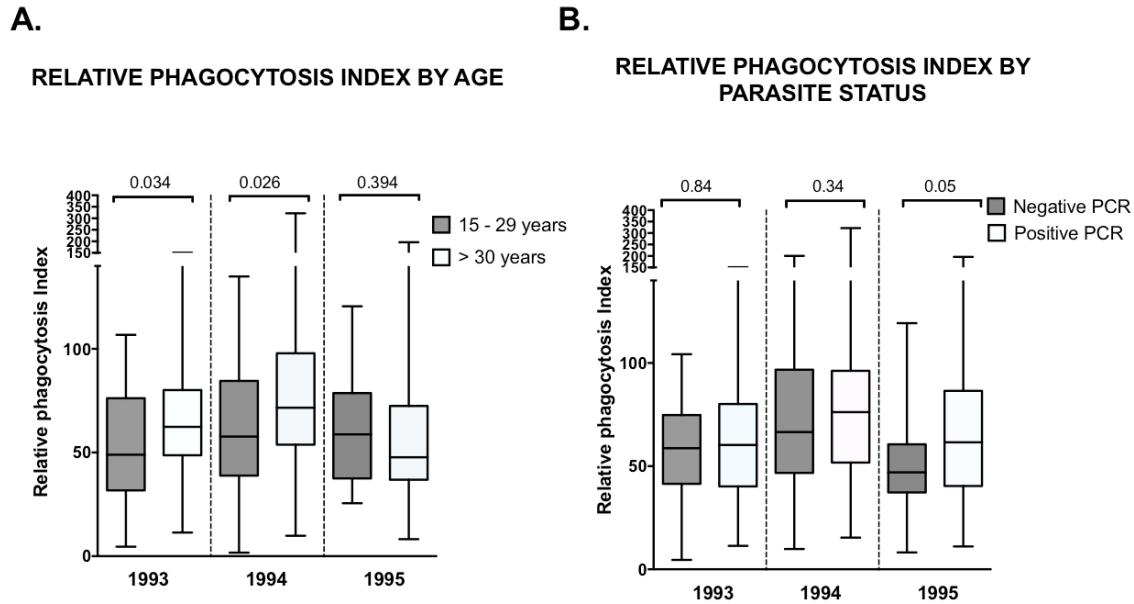


Figure 1: Relative phagocytosis indices in adults grouped by age and parasite status. (A) A comparison of the median phagocytosis indices in adults 30 years and older (white box and whisker plots) compared to 15-29 year olds in the 1993, 1994 and 1995 cross-sectional surveys. (B) Median phagocytosis indices in adults with asymptomatic parasitemia (as measured by qPCR of the 18S gene) compared to those who were parasite negative (black box and whisker plots). The relative phagocytosis indices were significantly higher in the older age group in the 1993 and 1994 cross-sectional survey while no significant difference were observed in the 1995 cross-sectional survey although a trend toward lower phagocytosis in older individuals was observed. The median RPI between adults aged 15 to 29 years versus those over 30 years and were as follows: a) 48.94 versus 62.36, p-value=0.034 b) 57.71 versus 71.62, p-value=0.026 and c) 58.74 versus 47.68, p-value=0.394 in the 1993, 1994 and 1995 cross-sectional surveys respectively. Similarly, the phagocytosis indices were compared in individuals who were parasite positive at the cross-sectional surveys as measured by qPCR detection of the 18S ribosomal RNA gene (B). In general, the indices were elevated in PCR positive individuals, although this difference was statistically significant in the 1995 cross-sectional survey only. The median RPI in parasite negative adults compared to parasite positive individuals were as follows: a) 58.69 versus 60.29, p-value=0.8479 b) 66.58 versus 76.19, p-value=0.3454 and c) 47.02 versus 61.58, p-value=0.05 in the 1993, 1994 and 1995 cross-sectional survey respectively.

## OPTIMISING PEMS COATING CONCENTRATION FOR C1q FIXING ANTIBODY ELISA

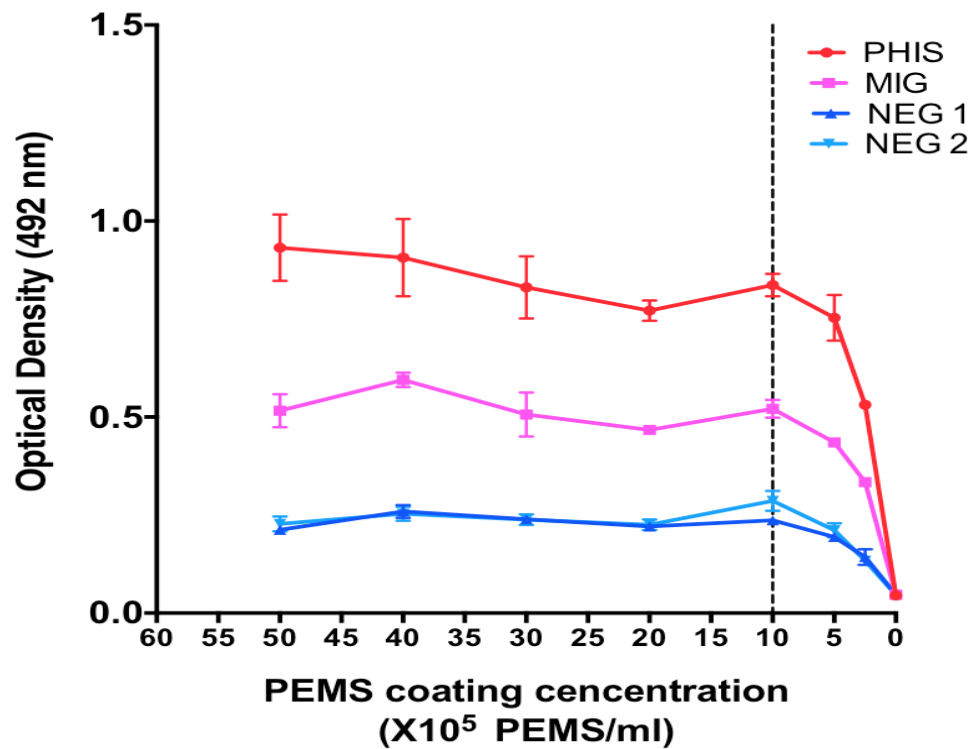


Figure 2: Optimizing the coating concentration of PEMS to measure antibody-mediated complement fixation on *P. falciparum* merozoites for longitudinal studies. A serial dilution of PEMS were coated and measured using a pool of sera from immune adults from Kilifi (PHIS: red line), purified immunoglobulins from Malawian adults (MIG: Pink line) and two serum samples from non-exposed Swedish adults (NEG: blue lines)

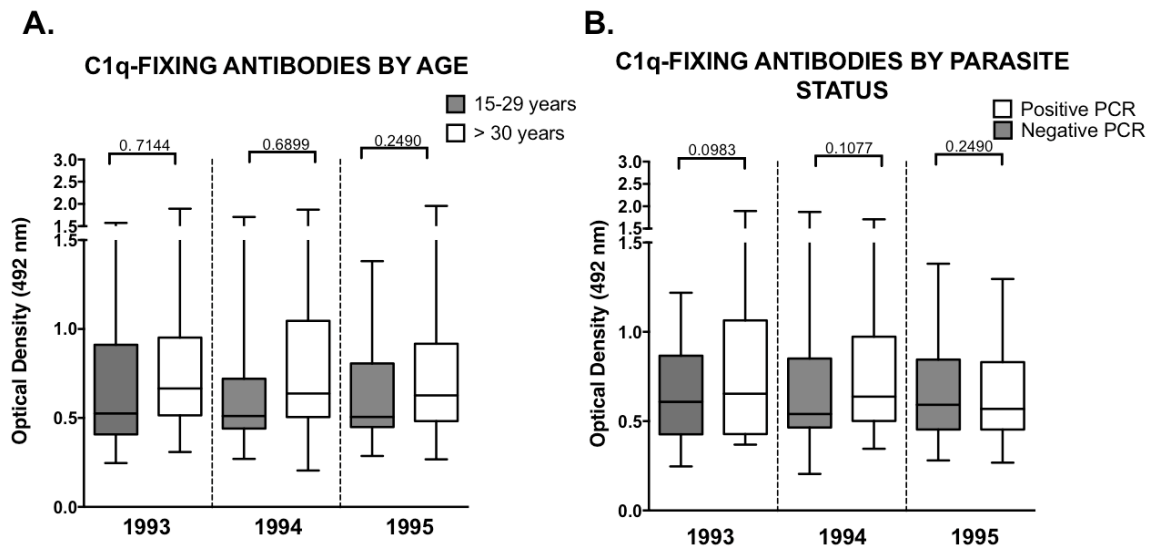


Figure 3: C1q-fixing antibodies in adults of the Nyamisati cohort by age and parasite status. (A) Median anti-C1q fixing antibodies were higher in adults 30 years (white box and whisker plots) and older compared to 15-29 year olds in all three cross-sectional surveys (black box and whisker plots). (B) Median anti-C1q fixing antibodies were higher in adults with asymptomatic parasitemia (as measured by qPCR of the 18S gene) than in those who were parasite negative (black box and whisker plots) in the 1993 and 1994 cross-sectional surveys. C1q-fixing antibody levels increased with age in all three cross-sectional surveys, although none of these were statistically significant. The median antibody levels in 15-29 year olds compared to 30 year olds were 0.525 versus 0.6535; p-value=0.098, 0.510 versus 0.591; p-value=0.107 and 0.506 versus 0.580; p-value=0.249 in the 1993, 1994 and 1995 surveys respectively. Similarly, C1q-fixing antibodies were elevated in adults with asymptomatic parasitemia (parasite positive) compared to those who were not in the 1993 and 1994 cross-sectional surveys, although these were not statistically significant.

## OPTIMISING PEMS COATING CONCENTRATION FOR ADRB ASSAY

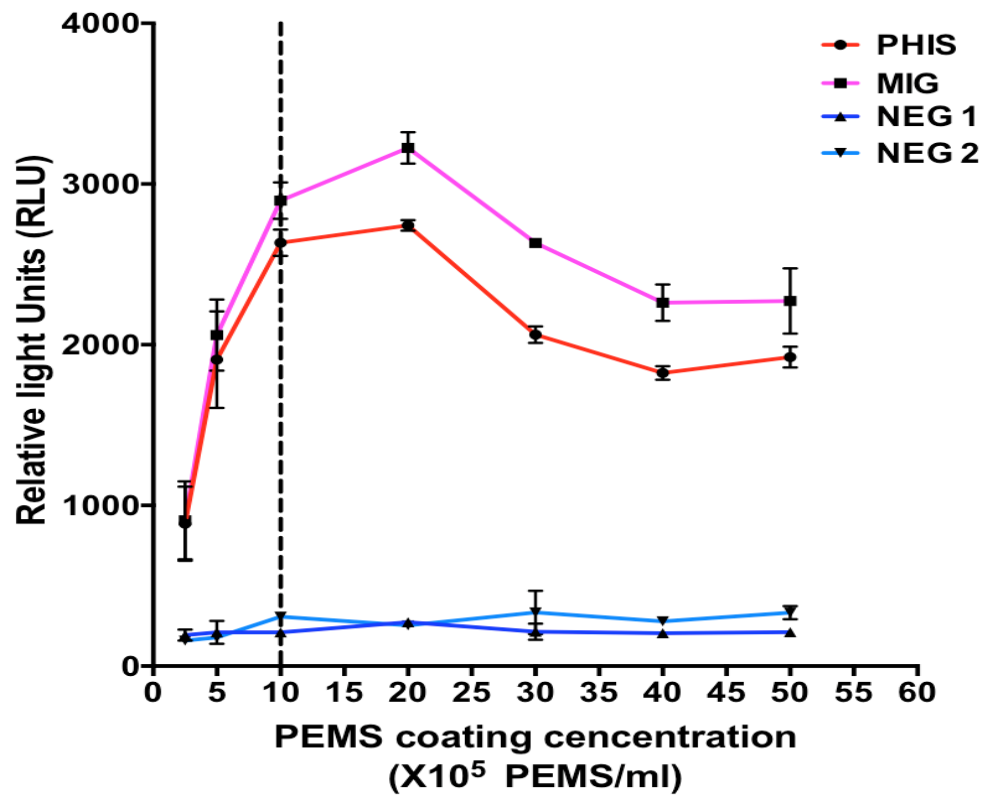


Figure 4: Optimizing the coating concentration of PEMS to measure antibody-dependent respiratory burst for longitudinal studies. A serial dilution of PEMS were coated and measured using a pool of sera from immune adults from Kilifi (PHIS: red line), purified immunoglobulins from Malawian adults (MIG: Pink line) and two serum samples from non-exposed Swedish adults (NEG: blue lines)

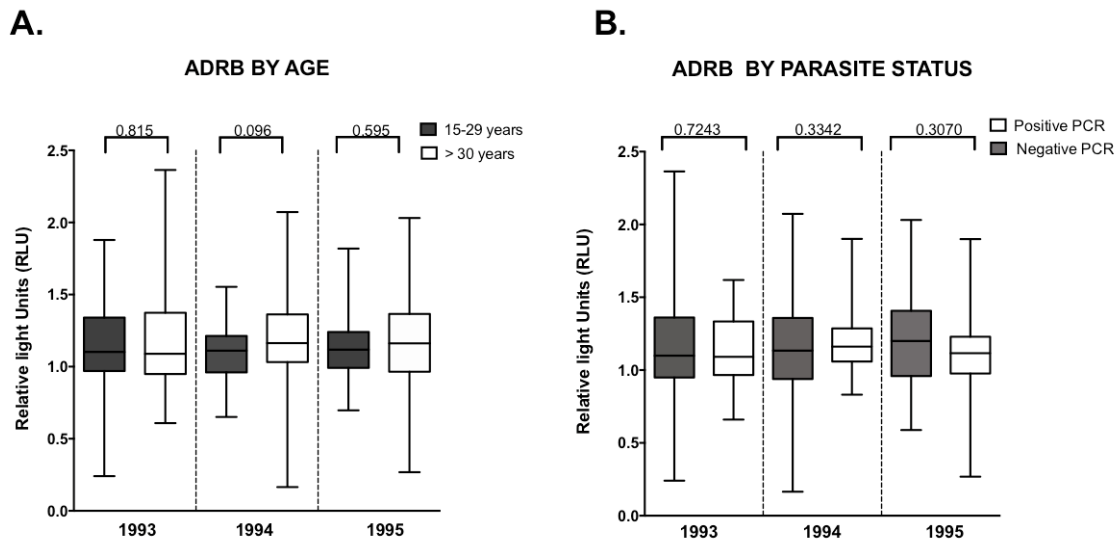


Figure 5: C1q-fixing antibodies in adults of the Nyamisati cohort by age and parasite status. (A) Median anti-C1q fixing antibodies were higher in adults 30 years (white box and whisker plots) and older compared to 15-29 year olds in all three cross-sectional surveys (black box and whisker plots). (B) Median anti-C1q fixing antibodies were higher in adults with asymptomatic parasitemia (as measured by qPCR of the 18S gene) than in those who were parasite negative (black box and whisker plots) in the 1993 and 1994 cross-sectional surveys. C1q-fixing antibody levels increased with age in all three cross-sectional surveys, although none of these were statistically significant. The median antibody levels in 15-29 year olds compared to 30 year olds were 0.525 versus 0.6535; p-value=0.097, 0.510 versus 0.591; p-value=0.106 and 0.506 versus 0.580; p-value=0.245 in the 1993, 1994 and 1995 surveys respectively. Similarly, C1q-fixing antibodies were elevated in adults with asymptomatic parasitemia (parasite positive) compared to those who were not in the 1993 and 1994 cross-sectional surveys, although these were not statistically significant.

## Appendix 6.2. Breadth of immune effector functions in adults who experienced a clinical episode versus those who did not.

Appendix 6.2: Lower breadth scores in individuals who experienced a clinical episode of malaria. The breadth of stable effector functions in 77 adults of the Nyamisati cohort.

Black squares indicate a stable response was detected while white squares indicate a non-responder to a specific effector function in the respective individual. Yellow highlights the individuals who experienced a clinical episode over the three year period.

Age	Clinical episode	ADRB	Opsonic phagocytosis	C1q-fixing	Breadth score
47	0				2
40	0				1
30	0				2
47	0				3
37	0				3
30	0				1
37	0				2
35	0				1
31	0				1
35	0				1
24	0				0
49	0				1
45	0				3
40	0				2
27	0				0
27	0				2
34	0				2
25	0				0
31	0				1
55	0				1
53	0				2
19	0				3
75	0				3
42	0				0
22	0				2
39	0				3
21	0				1
29	0				1
30	0				0
21	0				0
55	0				3
45	0				3
17	0				2
75	0				3
80	0				3
41	0				3
36	0				3
35	0				0
20	0				1
32	0				1
30	0				1
45	0				2
50	0				2
34	0				2
28	0				2
29	0				2
58	0				1
31	0				3
50	0				3
35	0				3
45	0				2
47	0				1
45	0				3
45	0				0
40	0				1
53	0				1
25	0				1
45	0				0
27	0				0
50	0				0
32	0				1
25	0				1
29	0				1
22	0				2
58	0				3
23	0				3
38	1				0
31	1				2
30	1				2
33	1				1
50	1				3
45	1				2
33	1				0
60	1				2
30	1				0
22	1				0
31	1				2



